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(54) Title: METHODS AND COMPOUNDS FOR MODULATING MELANOCORTIN RECEPTOR LIGAND BINDING AND ACTIVITY

(57) Abstract: This invention relates to methods and agonist/antagonist compounds for modulating melanocortin receptor-ligand binding. Also included is a method of identifying agonists and/or antagonists that bind to a ligand binding site for a melanocortin receptor of interest. Agonists and antagonists of ligand binding to melanocortin receptors also are provided. The invention is exemplified by identification and manipulation of the C-terminus of the human agouti related protein, which binds melanocortin receptors 3 and 4, and the prodution of AGRP peptidomimetics that are melanocortin receptor ligands. The methods can be applied to other melanocortin receptor agonists and antagonists.

METHODS AND COMPOUNDS FOR MODULATING MELANOCORTIN RECEPTOR LIGAND BINDING AND ACTIVITY

CROSS-REFERENCE TO RELATED APPLICATIONS

This claims benefit of and priority to U.S. provisional application no: 60/203,071, filed on May 9, 2000, and U.S. provisional application no: 60/226,047, filed on August 16, 2000, both of which are incorporated herein by reference, in their entirety, for all purposes.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

This invention was made with Government support under Grant Nos. GM46870, and DK58606 awarded by the National Institutes of Health. The Government of the United States of America may have certain rights in this invention.

FIELD OF THE INVENTION

The present invention relates to methods and compounds for modulating melanocortin receptor-ligand binding.

BACKGROUND OF THE INVENTION

The melanocortin (MC) receptors are a group of cell surface proteins that mediate a variety of physiological effects, including: regulation of adrenal gland function, such as production of glucocorticoid, cortisol and aldosterone; control of melanocyte growth and pigment production; hemoregulation; immunomodulation; and analgesia. Five distinct MC receptors have been cloned and are expressed in a variety of tissues, including melanocytes, adrenal cortex, brain, gut, placenta, skeletal muscle, lung, spleen, thymus, bone marrow, pituitary, gonads and adipose tissue. Two MC eceptors, MC3R and MC4R, are expressed in brain tissue, while MC1r is found in the skin. A variety of ligands termed melanocortins function as agonists that stimulate the activity of MC receptors. The melanocortins include melanocyte-stimulating hormones (MSH) such as alph-MSH, beta-MSH and gamma-MSH, as well as adrenocorticotropic hormone. Another MCR ligand that has been discovered is the Agouti Related Protein (AGRP), which is an antagonist.

AGRP is a natural antagonist of melanocortin receptors 3 and 4 (MC3R and MC4R). Recent biochemical investigations have identified AGRP as playing a major role in the regulation of mammalian feeding behavior. The human AGRP is a 132 residue polypeptide (SEQ ID NO:1) that is a naturally occurring competitive antagonist of melanocortin receptors 3 and 4 ("MC3r" and "MC4r"), the overexpression of which results in adult onset obesity and diabetes in mice (Shutter *et al.* (1997) *Genes Dev.* 11:593-602; Huszar *et al.* (1997) *Cell* 88:131-141; Hahn, *et al.* (1997) *Nature Neurosci.* 1:271-171).

AGRP binding to MC4r in particular is the subject of intense interest since knockout mice that do not express MC4r exhibit the same phenotype as caused by overexpression of AGRP (Huszar, *et al.*, *supra*). There is also evidence for the parallel expression of AGRP and neuropeptide Y in the arcuate nucleus of the hypothalamus, with neuropeptide Y known to stimulate feeding (Hahn, *et al.*, *supra*). This region of the brain also expresses MC4r and is involved in energy homeostasis. Research has also focused on other melanocortin receptors, their antagonists and methods for modulating receptor activity. See for example, Wei *et al.*, WO9943709.

The growing body of evidence linking AGRP to weight control has yet to elucidate its exact mechanism of action. However, studies on AGRP do benefit from analogy to the much more widely studied agouti protein, as AGRP was originally identified through the homology of its C-terminal region with the same region of the agouti protein (Shutter *et al., supra*). The agouti protein has been a focal point in obesity research for a number of years, since ectopic expression of the wild-type protein in mice leads to obesity and related disorders, *i.e.* the same symptoms as the overexpression of the more recently identified AGRP (Klebig, *et al.*, (1995) *Proc. Natl. Acad. Sci.* USA 92:4728-4732; Michaud, *et al.* (1997) *J. Endocrinol* 155:207-209. However, unlike AGRP, agouti has distinct expression patterns in mice and humans, making *in vivo* work with mice less applicable to human obesity disorders. AGRP, like agouti, is selective for MC3r and MC4r but has approximately 100-fold greater binding affinity than agouti at these receptors (Fong, *et al.* (1997) *Biochem. Res. Commun.* 237:629 611).

While full length agouti and AGRP are only 25% homologous, in their 46 residue Cys-rich C-terminal regions, nine of the 10 Cys residues are spatially conserved and there are a further 10 identical residues giving ~40% sequence identity. Three consecutive, conserved residues RFF (111-113 in human AGRP) were determined to be essential to the biological activity of both agouti (Kiefer, *et al.* (1997) *Biochemistry* 36:2084-2090; Kiefer,

et al. (1998) *Biochemistry* 37: 991-997) and AGRP (Tota *et al.* (1999) *Biochemistry* 38:897-904). Two recent investigations have shown that the chemically synthesized C-terminal region of AGRP competitively antagonizes α-melanocyte stimulating hormone (α-MSH) at melanocortin receptors with equal or greater potency than the full proteins (Quillan, *et al.* (1998) *FEBS Lett.* 428:59-62; Yang, *et al.* (1999) *Mol. Endocrinol.* 13:148-155), consistent with similar findings for agouti (Willard, *et al.* (1995) *Biochemistry* 34:12341-12346). Thus the Cys-rich C-terminal region of AGRP, is referred to as minimized agouti related protein ("MARP", $CVRLH_5ESCLG_{10}QQVPC_{15}CDPCA_{20}TCYCR_{25}FFNA\ F_{30}CYCR\ K_{35}LGTAM_{40}NPCSR_{45}T$, SEQ ID NO:2, where subscripts denote amino acid position).

The covalent structure of MARP exhibits five disulfide bonds, which exist between the following ten Cys residues (Bures, *et al.* (1988) *Biochemistry* 37:12172-12177): Cys_1 and Cys_{16}; Cys_8 and Cys_{22}; Cys_{15} and Cys_{33}; Cys_{19} and Cys_{43}; Cys_{24} and Cys_{31}.

Despite the important biological activities of AGRP, no experimental 3D structure has been available for this protein. The inhibitor cystine knot ("ICK") family of proteins are also disulfide-rich and the structures of these invertebrate toxins have been used to suggest possible structures for the agouti and AGRP C-terminal regions (Kiefer, *et al.* (1988) *Biochemistry* 37:991-997; Tota, *et al.*, *supra*). Indeed, the recently reported disulfide map for AGRP and a construct containing the C-terminal domain demonstrate ICK-like pairings (Norton, *et al.* (1998) *Toxicon* 36:1573-1583) of the 10 Cys residues: 1-16, 8-22, 15-33, 19-43, 24-31 (using MARP numbering, Bures, *et al.*, *supra*). Beyond such modeling, it is believed that the only structural data published on either agouti or AGRP are circular dichroism (CD) spectra which suggest that both proteins have little regular secondary structure, although there may be some β-sheet structure, consistent with ICK structural characteristics (Willard, *et al.*, *supra*; Rosenfeld, *et al.* (1998) *Biochemistry* 37:16041-16052).

U.S. Patents of interest in this area include: 5,994,087; 5,932,779; 5,869,257; 5,843,652; 5,817,787; 5,766,877; 5,731,408; 5,703,220; and 5,622,860. Also of interest are: WO 99/64002; WO 99/57148; WO 99/55679; WO 99/55832; WO 99/54358; WO 99/50295; WO 99/43709; WO 99/31508; WO 99/21571; WO 98/56914; WO 98/10068; and WO 97/43412.

SUMMARY OF THE INVENTION

This invention provides a detailed description of the three-dimensional (3D) structure (NMR structure) in solution of the human AGRP Cys-rich C-terminal region. In addition, further minimization of human AGRP is achieved and a minimal sequence (designated herein as MARP-33) is identified that provides both activity and receptor specificity against MC3r and MC4r. Information about this region permits design of compounds that bind to the ligand binding site of melanocortin receptors and modulate ligand binding to the receptor. The compounds include agonists and antagonists that modulate melanocortin receptor activity by promoting (agonists) or blocking (antagonists) ligand binding to the receptor and/or by activating the receptor themselves. In particular, using information provided herein, a class of peptidomimetics (*e.g.* non-peptide ligands) are provided that are ligands for the melanocortin receptor and that can block and/or modulate activity of the melanocortin receptors.

In one preferred embodiment, the peptides this emmbodiments provides a melanocortin receptor ligand that is a polypeptide comprising a peptide sequence having the formula: $CX^1X^2X^3X^4X^5X^6CX^7X^8X^9X^{10}X^{11}X^{12}CCDPX^{13}ATCYCX^{14}X^{15}X^{16}NAFCYCR_n$ (Formula I), where X^1, X^2, X^3, X^4, X^5, X^6, X^7, X^8, X^9, X^{10}, X^{11}, X^{12}, X^{13}, X^{14}, X^{15}, and X^{16} are independently selected amino acids, and n is zero or one. The amino acids include native amino acids, both D- and L-form amino acids, and modified or derivatized amino acids or amino acid analogues. In certain particularly preferred embodiments, X^1, X^2, X^3, X^4, X^5, X^6, X^7, X^8, X^9, X^{10}, X^{11}, X^{12}, X^{13}, X^{14}, X^{15}, and X^{16} are independently selected from the group consisting of alanine, asparagine, arginine,aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine. In certain embodimetns, the polypeptide consists of a polypeptide having the formula shown above (Formula I).

In certain preferred embodiments, the peptide is not AGRP and said polypeptide is not MARP. In certain preferred embodiments, the polypeptide excludes one or more of the final 13 residues of MARP (residues 34-46 of MARP). In certain embodiments, the polypeptide is not CVRLHESCLGQQVPCCDPAATCYCRFFNAFCYC (SEQ ID NO:3) or does not comprise this sequence.

In certain embodiments, $X^1X^2X^3X^4X^5X^6$ is VRLHES, or conservative substitutions thereof, and/or $X^7X^8X^9X^{10}X^{11}X^{12}$ is LGQQVP, or conservative substitutions

thereof, and/or $X^{14}X^{15}X^{16}$ is RFF or conservative substitutions thereof. In certain embodiments, X^{13} is not a cysteine and in particularly preferred embodiments, X^{13} is A.

In addition to the peptide melanocortin ligands described above, this invention provides a non-peptide melanocortin receptor ligand of the structural formula of Formula I shown herein in which B, U_1, U_2, R, R_1 and R_2 are independently selected from the group consisting of: hydrogen, alkyl, derivatized alkyl, cycloalkyl, derivatized cycloalkyl, halocycloalkyl, aloxycycloalkyl, heterocycloalkyl, aryl, arylalkyl, heteroaryl, and heteroarylalkyl; J is selected from the group consisting of carbon, nitrogen, silicon, and sulfur; X is selected from the group consisting of hydrogen, carbon, nitrogen, oxygen, silicon, and sulfur; and Z is selected from the group consisting of a continuing peptide bond, a hydroxyl; $-NH_2-$, -NH-(n), and -N-(n,n'), and -O-(y), where where n and n' are independently selected from the group consisting of alkyl, alkenyl, alkynyl, cycloalkyl, aryl, arylalkyl, heteroaryl, heteroarylalkyl, or a derivatized form thereof, and y is selected from the group consisting of alkyl, alkenyl, alkynyl, cycloalkyl, aryl, arylalkyl, heteroaryl heteroarylalkyl, or a derivatized form thereof. In preferred embodiments, the ligand is a ligand for a MC3r and/or an MC4r melanocortin receptor. Particularly preferred non-peptide ligands have a molecular weight ranging from about 200 to 1000 daltons. In preferred embodiments, the ligand has a structure that mimics the backbone of the AGRP active loop. Preferred ligands comprise a terminal gaunidino moiety and/or at least one methylbenzyl moiety. Particularly preferred ligands include the ligands of Formulas III, IV, V, and VI, shown herein.

In another embodiment, this invention also provides libraries for screening for modulators of a melanocortin receptor. Preferred libraries comprise a plurality of polypeptide and/or non-polypeptide members that are ligands (*e.g.* as described above) that bind to a melanocortin receptor (*e.g.* MC3r, MC4r, *etc.*). Preferred libraries comprise at least 10, more preferably at least 100, and most preferably at least 1000 different members. In certain embodiments, the libraries are provided in multi-well plates.

Also provided are pharmaceutical compositions comprising one or more of the melanocortin-binding peptide and/or non-peptide ligands of this invention (*e.g.* as described above). The pharmaceutical compositions optionally further comprise a pharmaceutically acceptable excipient.

In certain embodiments, this invention provides methods of modulating the activity of a melanocortin receptor (*e.g.* MC3r, MC4r, *etc.*) and/or methods of modulating a

melanocortin receptor mediated physiological process. The methods involve contacting the receptor with a peptide ligand and/or with a non-peptide ligand of this invention (*e.g.* as described above). In preferred embodiments, the contacting blockage of the receptor site and/or upregulation or downregulation of melanocortin receptor activity (*e.g.* as measured by cAMP assays). In certain embodiments the ligand is a melanocortin agonist. In certain embodiments, the ligand is a melanocortin receptor antagonist.

This invention also provides methods of prescreening for a modulator of a melanocortin receptor (*e.g.* MC3r, MC4r, *etc.*). The methods involve contacting a melanocortin receptor with one or more of the melanocortin receptor peptide ligands and/or non-peptide ligands of this invention (*e.g.* the ligands described above), and detecting binding of the peptide to the melanocortin receptor wherein specific binding of the peptide to the melanocortin receptor indicates that said peptide is a potential modulator of the melanocortin receptor.

In still another embodiment, this invention provides methods of screening for a modulator of melanocortin receptor activity. The methods involve contacting a melanocortin receptor (*e.g.* MC3r, MC4r, *etc.*) with a peptide ligand or a non-peptide ligand of this invention and detecting activity of the melanocortin receptor wherein a difference in activity of the receptor, as compared to a control, indicates that the ligand is a modulator of melanocortin receptor activity. In preferred embodiments, the control is a negative control comprising the same assay without the ligand.

This invention also provides a method of identifying a compound that modulates ligand binding to a melanocortin receptor, where the method involves modeling test compounds that fit spatially into a melanocortin receptor ligand binding site of interest using an atomic structural model of a melanocortin receptor binding region or portion thereof; screening the test compounds in an assay characterized by binding of a test compound to a melanocortin receptor ligand binding site; and identifying a test compound that modulates ligand binding to said melanocortin receptor. In preferred embodiments, the melanocortin receptor binding region comprises the minimized agouti related protein receptor binding region (mini-AGRP) or a portion thereof. In preferred embodiments, the atomic structural model comprises atomic coordinates of amino acid residues corresponding to residues 1-18 of the N-terminal loop of the minimized agouti related protein (residues 1-18 of SEQ ID NO:2), residues 19-13 of the central loop of the minimized agouti related protein (residues 19-34 of SEQ ID NO:2), and residues 35-46 of the C-terminal loop of the

minimized agouti related protein (residues 35-46 of SEQ ID NO:2). In certain preferred embodiments, the atomic structural model comprises atomic coordinates of amino acid residues corresponding to residues 19-34 of the central loop of the minimized agouti related protein (residues 19-34 of SEQ ID NO:2) and at least residues 15-18 of the N-terminal loop of the minimized agouti related protein (residues 15-18 of SEQ ID NO:2). In certain preferred embodiments, the atomic structural model comprises atomic coordinates of amino acid residues corresponding to residues 19-34 of the central loop of the minimized agouti related protein (residues 19-34 of SEQ ID NO:2) and at least 20% of the contiguous or non-contiguous residues or their atoms are selected from residues 1-18 of the N-terminal loop of the minimized agouti related protein (residues 1-18 of SEQ ID NO:2). The atomic structural model can comprises atomic coordinates of amino acid residues corresponding to residues 24-31 of the active loop of the minimized agouti related protein (residues 24-31 of SEQ ID NO:2). In certain embodiments, the atomic structural model comprises atomic coordinates of amino acid residues corresponding to residues 25-27 of the active loop of the minimized agouti related protein (residues 25-27 of SEQ ID NO:2). The screening is preferably *in vitro*. In certain embodiments, the screening is high throughput screening (HTS). Preferred assays include, but are not limited to biological assays. The test compound can be provided from a library of compounds and preferred test compounds are agonists or antagonists of ligand binding. Particularly preferred test compounds include a small organic molecule, a peptide, or peptidomimetic.

In another embodiment this invention provides methods for identifying an agonist or antagonist of ligand binding to a melanocortin receptor, said method comprising the steps of: providing the atomic coordinates of a melanocortin receptor binding region or portion thereof to a computerized modeling system; modeling compounds which match or mimic the receptor binding region and thus fit spatially into the melanocortin receptor ligand binding site; and identifying in an assay for melanocortin receptor activity a compound that increases or decreases the activity of said melanocortin receptor by binding the ligand binding site of said melanocortin receptor, whereby an agonist or antagonist of ligand binding is identified. In preferred embodiments, the melanocortin receptor binding region comprises the minimized agouti related protein receptor binding region or portion thereof.

Also provided is a machine-readable data storage medium, comprising a data storage material encoded with machine readable data which, when using a machine

programmed with instructions for using said data, is capable of displaying a graphical three-dimensional representation of a molecule that binds a melanocortin receptor comprising structure coordinates of amino acid residues corresponding to residues 1-18 of the N-terminal loop of the minimized agouti related protein (residues 1-18 of SEQ ID NO:2), residues 19-13 of the central loop of the minimized agouti related protein (residues 19-34 of SEQ ID NO:2), and residues 35-46 of the C-terminal loop of the minimized agouti related protein (residues 35-46 of SEQ ID NO:2), or a homologue of said molecule. In certain embodiments, the molecule is a melanocortin receptor agonist or antagonist. In particularly preferred embodiments, the molecule is defined by the set of structure coordinates depicted in Table 4 or Table 5, or a homologue of the molecule, the homologue having a root mean square deviation from the backbone atoms of said amino acids of not more than 10, preferably no more than 5 and most preferably no more than 2.54Å.

A machine-readable data storage medium is also provided that comprises a data storage material encoded with a first set of machine readable data that, when combined with a second set of machine readable data, using a machine programmed with instructions for using said first set of data and said second set of data, can determine at least a portion of the structure coordinates corresponding to the second set of machine readable data, wherein the first set of data comprises a Fourier transform of at least a portion of the structural coordinates selected from the group consisting of coordinates depicted in Table 4 or Table 5; and the second set of data comprises an X-ray diffraction pattern of a molecule.

In still another embodiment this invention provides an NMR structure of a minimized agouti related protein (mini-AGRP), embodied in a computer readable media.

This invention also provides a polypeptide comprising the amino acid sequence: CVRLHESCLGQQVPCCDPAATCYCRFFNAFCYC (SEQ ID NO:3) or a modified form thereof, where the polypeptide is not a full-length AGRP and the polypeptide is not a MARP. Preferred such polypeptides are chemically synthesized.

Also provided is a method of treating a disease state in mammals that is alleviated by treatment with a polypeptide having an amino acid sequence: CVRLHESCLGQQVP CCDPAATCYCRFFNAFCYC (SEQ ID NO:3) where the method comprises administering to a mammal in need of such a treatment a therapeutically effective amount of said polypeptide, or a pharmaceutically acceptable salt thereof. In certain embodiments, the disease state is a wasting syndrome. This invention also includes a pharmaceutical composition comprising a therapeutically effective amount of a polypeptide

of the sequence: CVRLHESCLGQQVPC CDPAATCYCRFFNAFCYC (SEQ ID NO:3) or a pharmaceutically acceptable salt thereof.

DEFINITIONS

The terms "residue" or "amino acid" as used herein refers to natural, synthetic, or modified amino acids (amino acid analogues). Such amino acids include both "D" and "L" forms. Various amino acid analogues include, but are not limited to, 2-aminoadipic acid, 3-aminoadipic acid, beta-alanine, beta-aminopropionic acid, 2-aminobutyric acid, 4-aminobutyric acid, piperidinic acid, 6-aminocaproic acid, 2-aminoheptanoic acid, 2-aminoisobutyric acid, 3-aminoisobutyric acid, 2-aminopimelic acid, 2,4- diaminobutyric acid, desmosine, 2,2'-diaminopimelic acid, 2,3-diaminopropionic acid, N-ethylglycine, N-ethylasparagine, hydroxylysine, allo-hydroxylysine, 3-hydroxyproline, 4-hydroxyproline, isodesmosine, allo-isoleucine, N-methylglycine, sarcosine, N-methylisoleucine, 6-N-methyllysine, norvaline, norleucine, ornithine, etc.

The terms "polypeptide", "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers. The term also includes variants on the traditional peptide linkage joining the amino acids making up the polypeptide.

The term "peptidomimetic" as used herein refers to a molecule that structurally and chemically resembles a peptide of two or more amino acids with respect to the features critical for a particular desired activity (e.g. receptor specificity, activity, etc.). The term "peptidomimetic" is includes peptide analogs that serve as appropriate substitutes for peptides in interactions with e.g., receptors and enzymes. Peptidomimetics can be "peptides" incorporating modified residues and/or backbones, e.g. that may have improved pharmacokinetic properties as a result of proteolytic stability, or unique structural and/or hydrogen bonding motifs, etc. Peptidomimetics also include organic molecules that are capapble of mimicing one more properties (e.g. binding specificity, affinity, etc.) of a peptide. Such organic molecule typically comprise a "scaffold" that mimics part or all of the 3-dimensional structure of the subject peptide and places appropriate functional groups in a spatial orientation sufficient to achieve the desired functional properties. Particularly preferred peptidomimetics include small organic molecules.

The term "small organic molecule" refers to a molecule of a size comparable to those organic molecules generally used in pharmaceuticals. The term excludes biological macromolecules (*e.g.*, proteins, nucleic acids, etc.). Preferred small organic molecules range in size up to about 5000 Da, more preferably up to 2000 Da, and most preferably up to about 1000 Da.

The term database refers to a means for recording and retrieving information. In preferred embodiments the database also provides means for sorting and/or searching the stored information. The database can comprise any convenient media including, but not limited to, paper systems, card systems, mechanical systems, electronic systems, optical systems, magnetic systems or combinations thereof. Preferred databases include electronic (*e.g.* computer-based) databases. Computer systems for use in storage and manipulation of databases are well known to those of skill in the art and include, but are not limited to "personal computer systems", mainframe systems, distributed nodes on an inter- or intra-net, data or databases stored in specialized hardware (*e.g.* in microchips), and the like.

The term "conservative substitution" is used in reference to proteins, peptides, *etc.* to reflect amino acid substitutions that do not substantially alter the activity (specificity or binding affinity) of the molecule. Typically, conservative amino acid substitutions involve substitution of one amino acid for another amino acid with similar chemical properties (*e.g.* charge or hydrophobicity). The following six groups each contain amino acids that are typical conservative substitutions for one another: 1) Alanine (A), Serine (S), Threonine (T); 2) Aspartic acid (D), Glutamic acid (E); 3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (K); 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

The terms "binding partner", or "capture agent", or a member of a "binding pair" refers to molecules that specifically bind other molecules to form a binding complex such as antibody-antigen, lectin-carbohydrate, nucleic acid-nucleic acid, biotin-avidin, etc.

The term "specifically binds", as used herein, when referring to a biomolecule (*e.g.*, protein, nucleic acid, antibody, receptor, etc.), refers to a binding reaction which is determinative of the presence biomolecule in heterogeneous population of molecules (*e.g.*, proteins and other biologics). Thus, under designated conditions (*e.g.* immunoassay conditions in the case of an antibody or stringent hybridization conditions in the case of a nucleic acid), the specified ligand or antibody binds to its particular "target"

molecule and does not bind in a significant amount to other molecules present in the sample.

"Alkyl" means a linear saturated monovalent hydrocarbon radical of one to six carbon atoms or a branched saturated monovalent hydrocarbon radical of three to six carbon atoms, *e.g.*, methyl, ethyl, propyl, 2-propyl, butyl, pentyl, and the like.

"Alkenyl" means a linear monovalent hydrocarbon radical of two to six carbon atoms or a branched monovalent hydrocarbon radical of three to six carbon atoms containing at least one double bond, *e.g.*, ethenyl, 2-propenyl, and the like.

"Alkynyl" means a linear monovalent hydrocarbon radical of two to six carbon atoms or a branched monovalent hydrocarbon radical of three to six carbon atoms containing at least one triple bond, *e.g.*, ethynyl, propynyl, butynyl, and the like.

"Cycloalkyl" means a cyclic saturated monovalent hydrocarbon radical of three to seven carbon atoms, *e.g.*, cyclopropyl, cyclohexyl, and the like.

"Halo" means fluoro, chloro, bromo, and iodo.

"Haloalkyl" means alkyl substituted with one or more halogen atoms, including those substituted with different halogens, *e.g.*,-CH_2Cl, --CF_3, --CH_2CF_3, --CF_2CF_3, --CH_2CCl_3, and the like.

"Alkoxy", "alkenyloxy", "cycloalkyloxy", or "haloalkyloxy" means a radical --OR where R is alkyl, alkenyl, cycloalkyl, or haloalkyl respectively as defined above, *e.g.*, methoxy, ethoxy, propoxy, 2-propoxy, ethenyloxy, cyclopropyloxy, cyclobutyloxy, --OCH_2 Cl, --OCF_3, and the like.

"Alkylthio" or "cycloalkylthio" means a radical --SR where R is alkyl or cycloalkyl respectively as defined above, *e.g.*, methylthio, butylthio, cyclopropylthio, and the like.

"Acyl" means a radical --C(O)R where R is hydrogen, alkyl, or haloalkyl as defined above, *e.g.*, formyl, acetyl, trifluoroacetyl, butanoyl, and the like.

"Amino" means a radical --NH_2

"Monosubstituted amino" means a radical --NHR where R is alkyl or acyl, *e.g.*, methylamino, (1-methylethyl)amino, and the like.

"Disubstituted amino" means a radical --NRR' where R and R' are independently alkyl or acyl, *e.g.*,dimethylamino, methylethylamino, di(1-methylethyl)amino, and the like.

"Hydroxyalkyl" means a linear monovalent hydrocarbon radical of two to six carbon atoms or a branched monovalent hydrocarbon radical of three to six carbons substituted with one or two hydroxy groups, provided that if two hydroxy groups are present they are not both on the same carbon atom. Representative examples include, but are not limited to, 2-hydroxyethyl, 2-hydroxypropyl, 3-hydroxypropyl, 1-(hydroxymethyl)-2-methylpropyl, 2-hydroxybutyl, 3-hydroxybutyl, 4hydroxybutyl, 2,3-dihydroxypropyl, 1-(hydroxymethyl)-2-hydroxyethyl, 2,3-dihydroxybutyl, 3,4dihydroxybutyl and 2-(hydroxymethyl)-3-hydroxypropyl, 2-hydroxyethyl, 2,3-dihydroxypropyl, and 1-(hydroxymethyl)-2-hydroxyethyl.

"Alkoxyalkyl" means a linear monovalent hydrocarbon radical of one to six carbon atoms or a branched monovalent hydrocarbon radical of three to six carbons substituted with at least one alkoxy group as defined above, *e.g.*, 2-methoxyethyl, 2-methoxypropyl, and the like.

"Hydroxyalkyloxy" or "alkoxyalkyloxy" means a radical-OR where R is hydroxyalkyl or alkoxyalkyl respectively as defined above, *e.g.*, 2-hydroxyethyloxy, 2-methoxyethyloxy, and the like.

"Aminoalkyl" means a linear monovalent hydrocarbon radical of two to six carbon atoms or a branched monovalent hydrocarbon radical of three to six carbons substituted with at least one --NRR' where R and R' are independently selected from hydrogen, alkyl, or acyl, *e.g.*, 2-aminoethyl, 2-N,N-diethylaminopropyl, 2-N-acetylaminoethyl, and the like.

"Aryl" means a monovalent monocyclic or bicyclic aromatic hydrocarbon radical of 6 to 12 ring atoms, and optionally substituted independently with one or more substituents selected from alkyl, haloalkyl, cycloalkyl, alkoxy, alkylthio, halo, nitro, acyl, cyano, amino, monosubstituted amino, disubstituted amino, -hydroxy, carboxy, or alkoxycarbonyl. Representative examples include, but are not limited to, phenyl, biphenyl, 1-naphthyl, and 2-naphthyl and the derivatives thereof.

"Heteroaryl" means a monovalent monocyclic or bicyclic aromatic radical of 5 to 10 ring atoms containing one or more, sometimes one or two ring heteroatoms selected from N, O, or S, the remaining ring atoms being C. The heteroaryl ring is optionally substituted independently with one or more substituents, sometimes one or two substituents, selected from alkyl, haloalkyl, cycloalkyl, alkoxy, alkylthio, halo, nitro, acyl, cyano, amino, monosubstituted amino, disubstituted amino, hydroxy, carboxy, or alkoxycarbonyl.

Specifically the term heteroaryl includes, but is not limited to, pyridyl, pyrrolyl, thienyl, furanyl, indolyl, quinolyl, benzopyranyl, and thiazolyl, and the derivatives thereof.

"Heterocycloamino" means a saturated monovalent cyclic group of 3 to 8 ring atoms, wherein at least one ring atom is N and optionally contains a second ring heteroatom selected from the group consisting of N, O, or $S(O)_n$ (where n is an integer from 0 to 2), the remaining ring atoms being C. The heterocycloamino ring may be optionally fused to a benzene ring or it may be optionally substituted independently with one or more substituents, sometimes one or two substituents, selected from alkyl, haloalkyl, cycloalkyl, cycloalkylalkyl, aryl, aralkyl, heteroaryl, heteroaralkyl, halo, cyano, acyl, amino, monosubstituted amino, disubstituted amino, carboxy, or alkoxycarbonyl. More specifically the term heterocycloamino includes, but is not limited to, pyrrolidino, piperidino, morpholino, piperazino, indolino, and thiomorpholino, and the derivatives thereof.

"Heterocyclo" means a saturated monovalent cyclic group of 3 to 8 ring atoms in which one or two ring atoms are heteroatoms selected from N, O, or $S(O)_n$, where n is an integer from 0 to 2, the remaining ring atoms being C. The heterocyclo ring may be optionally fused to a benzene ring or it may be optionally substituted independently with one or more substituents, sometimes one or two substituents, selected from alkyl, haloalkyl, cycloalkyl, cycloalkylalkyl, aryl, aralkyl, heteroaralkyl, halo, cyano, acyl, monosubstituted amino, disubstituted amino, carboxy, or alkoxycarbonyl. More specifically the term heterocyclo includes, but is not limited to, pyrrolidino, piperidino, morpholino, piperazino, tetrahydropyranyl, and thiomorpholino, and the derivatives thereof.

"Cycloalkylalkyl" means a radical $-R^aR^b$ where R^a is an alkylene group and R^b is a cycloalkyl group as defined above *e.g.*, cyclopropylmethyl, cyclohexylpropyl, 3-cyclohexyl-2-methylpropyl, and the like.

"Cycloalkylalkyloxy" means a radical --OR where R is a cycloalkylalkyl group as defined above *e.g.*, cyclopropylmethyloxy, 3-cyclohexylpropyloxy, and the like.

"Aralkyl" means a radical - $R^a R^b$ where R^a is an alkylene group and R^b is an aryl group as defined above *e.g.*, benzyl, phenylethyl, 3-(3-chlorophenyl)-2-methylpentyl, and the like.

"Heteroaralkyl" means a radical - $R^a R^b$ where R^a is an alkylene group and R^b is a heteroaryl group as defined above *e.g.*, 2-,3-, or 4-pyridylmethyl, furan-2-ylmethyl and the like.

"Heterocycloalkyl" means a radical - R^aR^b where R^a is an alkylene group and R^b is a heterocyclo group as defined above *e.g.*, morpholin-4-ylethyl, tetrahydrofuran-2-ylmethyl and the like.

A "pro-drug" is a compound that releases an active drug (*e.g.* a mini-AGRP, a peptidomimetic as described herein) when such prodrug is administered to a mammalian subject. In certain embodiments, prodrugs are prepared by modifying functional groups present in the peptidomimetics of this invention or by modifyng mini-AGRP polypeptides in such a way that the modifications may be cleaved *in vivo* to release the active compound. Peptidomimetic prodrugs include the peptideomimetics described herein wherein a hydroxy, amino, or sulfhydryl group in compound is bonded to any group that may be cleaved *in vivo* to regenerate the free hydroxyl, amino, or sulfhydryl group, respectively. Examples of prodrugs include, but are not limited to esters (*e.g.*, acetate, formate, and benzoate derivatives), carbamates (*e.g.*, N,N-dimethylaminocarbonyl) of hydroxy functional groups in compounds of formula (I), and the like.

"Optional" or "optionally" means that the subsequently described event or circumstance may, but need not, occur, and that the description includes instances where the event or circumstance occurs and instances in which it does not. For example, "heterocyclo group optionally mono- or di- substituted with an alkyl group" means that the alkyl may, but need not, be present, and the description includes situations where the heterocyclo group is mono- or disubstituted with an alkyl group and situations where the heterocyclo group is not substituted with the alkyl group.

Compounds that have the same molecular formula but differ in the nature or sequence of bonding of their atoms or the arrangement of their atoms in space are termed "isomers." Isomers that differ in the arrangement of their atoms in space are termed "stereoisomers." Stereoisomers that are not mirror images of one another are termed "diastereomers" and those that are non-superimposable mirror images of each other are termed "enantiomers." When a compound has an asymmetric center, for example, it is bonded to four different groups, a pair of enantiomers is possible. An enantiomer can be characterized by the absolute configuration of its asymmetric center and is described by the R- and S-sequencing rules of Cahn and Prelog, or by the manner in which the molecule rotates the plane of polarized light and designated as dextrorotatory or levorotatory (*i.e.*, as (+) or (-)-isomers respectively). A chiral compound can exist as either individual

enantiomer or as a mixture thereof. A mixture containing equal proportions of the enantiomers is called a "racemic mixture."

The compounds of this invention may possess one or more asymmetric centers; such compounds can therefore be produced as individual (R)- or (S)- stereoisomers or as mixtures thereof. Unless indicated otherwise, the description or naming of a particular compound in the specification and claims is intended to include both individual enantiomers and mixtures, racemic or otherwise, thereof. The methods for the determination of stereochemistry and the separation of stereoisomers are well-known in the art (see discussion in Chapter 4 of "Advanced Organic Chemistry", 4th edition J. March, John Wiley and Sons, New York, 1992).

A "pharmaceutically acceptable excipient" means an excipient that is useful in preparing a pharmaceutical composition that is generally safe, non-toxic and neither biologically nor otherwise undesirable, and includes an excipient that is acceptable for veterinary use as well as human pharmaceutical use. "A pharmaceutically acceptable excipient" as used in the specification and claims includes both one and more than one such excipient.

A "pharmaceutically acceptable salt" of a compound means a salt that is pharmaceutically acceptable and that possesses the desired pharmacological activity of the parent compound. Such salts include: (1) acid addition salts, formed with inorganic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid, and the like; or formed with organic acids such as acetic acid, propionic acid, hexanoic acid, cyclopentanepropionic acid, glycolic acid, pyruvic acid, lactic acid, malonic acid, succinic acid, malic acid, maleic acid, fumaric acid, tartaric acid, citric acid, benzoic acid, 3-(4-hydroxybenzoyl)benzoic acid, cinnamic acid, mandelic acid, methanesulfonic acid, ethanesulfonic acid, 1,2-ethanedisulfonic acid, 2-hydroxyethanesulfonic acid, benzenesulfonic acid, 4-chlorobenzenesulfonic acid, 2-naphthalenesulfonic acid, 4-toluenesulfonic acid, camphorsulfonic acid, 4methylbicyclo›2.2.2!oct-2-ene-1-carboxylic acid, glucoheptonic acid, 4,4'-methylenebis-(3-hydroxy-2-ene-1-carboxylic acid), 3-phenylpropionic acid, trimethylacetic acid, tertiary butylacetic acid, lauryl sulfuric acid, gluconic acid, glutamic acid, hydroxynaphthoic acid, salicylic acid, stearic acid, muconic acid, and the like; or (2) salts formed when an acidic proton present in the parent compound either is replaced by a metal ion, *e.g.*, an alkali metal ion, an alkaline earth ion, or an

aluminum ion; or coordinates with an organic base such as ethanolamine, diethanolamine, triethanolamine, tromethamine, N-methylglucamine, and the like.

"Treating" or "treatment" of a disease includes: (1) preventing the disease, *i.e.* causing the clinical symptoms of the disease not to develop in a mammal that may be exposed to or predisposed to the disease but does not yet experience or display symptoms of the disease, (2) inhibiting the disease, *i.e.*, arresting or reducing the development of the disease or its clinical symptoms, or (3) relieving the disease, *i.e.*, causing regression of the disease or its clinical symptoms.

A "therapeutically effective amount" means the amount of a compound that, when administered to a mammal for treating a disease, is sufficient to effect such treatment for the disease. The "therapeutically effective amount" will vary depending on the compound, the disease and its severity and the age, weight, etc., of the mammal to be treated.

The term database refers to a means for recording and retrieving information. In preferred embodiments the database also provides means for sorting and/or searching the stored information. The database can comprise any convenient media including, but not limited to, paper systems, card systems, mechanical systems, electronic systems, optical systems, magnetic systems or combinations thereof. Preferred databases include electronic (*e.g.* computer-based) databases. Computer systems for use in storage and manipulation of databases are well known to those of skill in the art and include, but are not limited to "personal computer systems", mainframe systems, distributed nodes on an inter- or intra-net, data or databases stored in specialized hardware (*e.g.* in microchips), and the like

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B show the Cα backbone of the MARP minized average structure determined at 500 mHz and 800 mHz, respectively. Figure 1A: The N-terminal loop, central loop and C-terminal loop are indicated. Disulfide bonds are represented by dashed lines. Spheres represent residues with amides protected from HX for more than 12 hours, more than 24 hours and more than 8 days. Figure 1B shows a C(alpha) backbone representation of the lowest energy AGRP(87-132) structure of the family of 40 determined from 800 MHz NOESY data and deposited in the PDB (code: 1HYK). The N-terminal loop is show in light grey at the lower left,the central loop is dark grey (top middle), and the C-terminal loop is shown at the lower right. The disulfide bonds are included as thin dashed

white lines. The spheres highlight residues the amides of which were protected from HX: medium gray > 12 hours, light gray > 24 hours and dark gray > 8 days.

Figure 2A depicts the heavy atom (non-hydrogen) backbone representation of MARP for 14 NMR structures with residues 1-34 fit to the minimized average structure (Root Mean Square Deviation, "RMSD" 1.49Å). Only the minimized average structure (thick cylinder) is shown for the more disordered C-terminal loop. Figure 2B shows an all atom backbone representation of AGRP(87-132) for the 40 structures deposited in the PDB calculated from 800 MHz NOESY data. Only the backbone atoms of 1-34 have been aligned (RMSD = 0.34 angstroms). The disulfide-bound residues are indicated with straight lines interconnecting regions of the backbone, and are included for all members of the family.

Figure 3 shows the backbone atoms for residues 24-31 of the family of 20 structures with residues 24-31 fit to the minimized average structure. The side chain heavy atoms of residues 25, 26 and 27, essential for activity, are shown.

Figure 4 illustrates a reaction scheme for the synthesis of peptidomimetics of this invention.

DETAILED DESCRIPTION

The agouti related protein ("AGRP") is a mammalian signaling molecule, involved in weight homeostasis, that causes adult onset obesity when overexpressed in mice. AGRP was originally identified by homology to the agouti protein, another potent signaling molecule involved in obesity disorders in mice. While AGRP's exact mechanism of action is unknown, it has been identified as a competitive antagonist of melanocortin receptors 3 and 4 ("MC3r" and "MC4r"). MC4r in particular is implicated in the hypothalamic control of feeding behavior. Full length agouti and AGRP are only 25% homologous, however, their active C-terminal regions are ~40% homologous, with nine out of the 10 Cys residues spatially conserved. Until now, 3D structures have not been available for either agouti, AGRP or their C-terminal regions.

In one embodiment, this invention provides a detailed three-dimensional (3D) NMR structure in solution of the human AGRP Cys-rich C-terminal region as determined by ^{1}H NMR using a protein prepared by total chemical synthesis. As used herein the term "the NMR structure" is understood to refer to the minimized average of the family of NMR structures. Because biochemical investigations demonstrate that this

minimal region retains full biological activity, this protein is referred to herein as minimized agouti related protein ("MARP"). MARP residues 1-46 (SEQ ID NO:2), correspond to human AGRP residues 87-132 (residues 87-132 of SEQ ID NO:1). Thus, human AGRP numbering is obtained by adding 86 to MARP numbering.

MARP's topology is characterized by three large loops (referred to herein as the N-terminal loop, the central loop and the C-terminal loop), with four of the five disulfide bridges at the base of the structure, and an absence of canonical secondary structure such as helices or sheets. Two of the three loops are structurally well characterized by the NMR data as indicated by low RMSDs. The region of MARP containing the RFF triplet (Tota, et al., *supra*) (residues 25-27 in MARP) necessary for function is located in one of the best defined regions of the protein. While previously reported structural models of the C-terminal region of AGRP were attempted based on Cys homology between AGRP and certain toxin proteins, Cys spacing was not sufficient to correctly determine the 3D fold of the molecule. It was initially thought, based on 500 mHz data that MARP did not adopt an ICK-like fold, however, new data obtained at 800 mHZ (Table 5 herein) shows that MARP does adopt the ICK-like fold.

The 3D structure of MARP provided herein presents a basis for the development of methods and compositions for identifying compounds that modulate melanocortin receptor activity, in particular the activity of MC3r and MC4r. The 3D structure presents precise structural information that permits the rational design of compounds that preferentially modulate MC4r or MC3r activity.

Based on an analysis of this structure, a minimal AGRP domain is identified (designated as a mini-AGRP) that shows both melanocortin activity and melanocortin specificity. Residues are identified that can be systematically or randomly altered to produce a large number of mini-AGRPs having differing receptor specificity and/or binding affinity.

Collections of such mini-AGRPs provide convenient libraries that can be screened to identify mini-AGRPs having particular activity/specificity profiles. THese AGRPs can be used to modulate melanocortin receptor activity (*in vivo* or *in vitro*) or they can be used as "lead compounds" for the design of peptido mimetics.

In addition a class of peptidomimetics is identified herein that are specific ligands to melanocortin receptors (*e.g.* MC3r and/or MC4r). In certain embodiments, the peptidomimetics are used to modulate melanocortin receptor activity *in vivo* or *in vitro*. In

other embodiments, they are provided as libraries that can be screened for peptidomimetics having particular desired specificity/activity profiles.

I. Structure of MARP

The 3D structure of MARP is characterized by three loops held together at the base by an apparent scaffold of four disulfide bonds 1-16, 8-22, 15-33 and 19-43. The fifth disulfide bond, 24-31, further stabilizes the base of the active loop which presents the RFF triplet on the protein surface. There is no identifiable canonical helical structure. The 800 mHz data reveal a small beta sheet structure. The RFF triplet is critical for the activity of MARP as a competitive antagonist of α-MSH stimulated activation of MC4r signaling. The structure described herein shows that MARP is structured to present the side chains of the RFF triplet on the surface of the protein and to the surrounding solvent. Recent work demonstrates that MARP is much more active than smaller AGRP derived peptides containing the RFF triplet (Tota, et al. (1999) *Biochemistry* 38:897-904). Thus, the detailed fold of the central loop and perhaps the presence of the N- and C-terminal loops are important for AGRP function. In addition, based upon work with chimeras of melanocortin receptors, we believe the N- and C-terminal loops confer receptor subtype specificity.

The previous absence of structural data on both AGRP and agouti encouraged the modeling of the C-terminal regions of these proteins onto the ICK family (Norton, et al., *supra*)) which is characterized by homologous Cys spacing (Kiefer, et al., *Biochemistry* 37:991-997 (1998); Tota, et al., *supra*). The ICK family of proteins primarily consists of small (<60 residues) disulfide-rich (three or four disulfides) toxin proteins from the venom of spiders and cone snails, which function as ion channel antagonists (Norton, et al., *supra*). The coincidence between the function of the majority of these toxins and the recent description of part of the agouti protein's mechanism of action being calcium dependent (Kim, et al., *FASEB J.* 10:1646-1652 (1996); Kim, et al., *Am. J. Physiol.* 272:E379-384 (1997); Jones, et al., *Am. J. Physiol.* 270:E192-196 (1996)) further encouraged these homology modeling efforts. The ICK motif in particular is characterized by the topology of the three disulfide bonds corresponding to 1-16, 8-22 and 15-33 in MARP. In the ICK motif the first two disulfide bonds with their intervening main chain atoms form a topological circle through which the third disulfide bond passes, forming the *cystine knot* (Norton, et al., *supra*). The motif is further characterized by the identification of an irregular triple stranded antiparallel β-sheet, roughly corresponding to residues 6-8,

20-24 and 31-34 in MARP. The remaining two disulfide bonds in MARP each occur in individual ICK proteins as separate examples of potential "non-motif" disulfide bonds, although no examples of ICK motif proteins with five disulfide bonds have been observed.

Despite these apparent similarities, the experimental structure of MARP determined at 500 mHz, suggested that this protein did not satisfy the criteria required for inclusion in the ICK family. While the first two disulfide bonds in MARP 1-16 and 8-22, together with the polypeptide backbone form a topological circle, none of the remaining disulfides passes through the circle to form a cystine knot. Instead, disulfide bond 15-33 is positioned adjacent to the circle with all of the fold on one side of this circle. In addition, MARP lacks the β-sheet found in ICK family proteins. The experimental determination of the distinctive 3D structure of MARP described herein suggests that Cys spacing and even the disulfide map of small Cys-rich proteins may not always be sufficient to accurately predict protein folds. These results speak to the potential limitations of "homology modeling" of protein structures, and may have important implications for the emerging field of genomic structural biology. However, based on the 800 mHz NMR data, we have not assigned MARP to the ICK class.

As described in the Examples, ligand binding studies, and analysis of atomic models derived from the MARP NMR structure reveal for the first time a previously unknown structure for MARP and its receptor binding region. By "receptor binding region" is intended a structural segment or segments of melanocortin receptor ligands, and MARP in particular, folded in such a way so as to give the proper geometry and amino acid residue conformation for binding to a melanocortin receptor. By "ligand binding site" is intended a structural segment or segments of melanocortin receptor polypeptide chain folded in such a way so as to give the proper geometry and amino acid residue conformation for binding a ligand. These are the physical arrangement of protein atoms in three-dimensional space forming a receptor binding region or a ligand binding site pocket or cavity.

The MARP structure has three major loops: the N-terminal loop, residues 1-18 (residues 1-18 of SEQ ID NO:2), the central loop, residues 19-34 (residues 19-34 of SEQ ID NO:2) and the C-terminal loop, residues 35-46 (residues 35-46 of SEQ ID NO:2). Residues forming the receptor binding region are amino acids corresponding to (*i.e.*, the same as or equivalent to) residues 24-31 of the central loop (residues 24-31 of SEQ ID

NO:2), referred to herein as the "active" loop. In particular, residues 25, 26 and 27 (the "RFF" triplet) of the active loop are critical for activity.

It has been found that a polypeptide comprising the central loop, preferably at least a portion of the N-terminal loop, is desirable for optimal biological activity. As used herein, the term "at least a portion of the N-terminal loop" is intended to mean a sequence that corresponds to (*i.e.*, the same as or equivalent to), at least residues 15 to 18 of the N-terminal loop (residues 15-18 of SEQ ID NO:2), preferably at least residues 8 to 18 of the N-terminal loop (residues 8-18 of SEQ ID NO:2), and is also intended to include all of the N-terminal loop, *i.e.*, residues 1 to 18 of the N-terminal loop (residues 1-18 of SEQ ID NO:2). The term is also intended to mean a sequence that corresponds to at least 20%, preferably at least 60%, and more preferably at least 90%, of the contiguous or non-contiguous amino acid residues or their atoms selected from amino acid residues 1 to 18 of the N-terminal loop (residues 1-18 of SEQ ID NO:2).

II. Structure of mini-AGRP

Another aspect of the invention also pertains to the identification of "minimal" polypeptides (mini-AGRPs) that show specific binding and/or activity at melanocortin receptors.show. Certain preferred mini-AGRPs are strong antagonists of melanocortin receptor types 3 and 4, and are useful for antagonizing melanocortin receptor in a variety of contexts, *e.g.* for the treatment of eating disorders and obesity. As indicated above, research has demonstrated that the final 46 residues of the human AGRP sequence ("MARP"), possesses full receptor antagonist activity. The structure of MARP, solved by use of nuclear magnetic resonance and described herein, indicated that the final 13 residues of MARP are unstructured are unlikely to play a significant role in receptor binding or antagonism. Thus, substantial further minimization of human AGRP was achieved. Specifically, the sequence (indicated by the single letter amino acid code): $CVRLH_5ESCLG_{10}QQVPC_{15}CDP\underline{A}A_{20}TCYCR_{25}FFNAF_{30}CYC$ (SEQ ID NO:3) designated "MARP-33" (based upon its 33 amino acid length) is believed to fold with proper native-like disulfide bonds and possesses the full biological activity of MARP. This sequence is equivalent to the first 33 amino acids of MARP but contain a Cys to Ala substitution at position 19 (indicated as "A") to avoid having a non-bridged thiol.

Another mini-AGRP was made having the sequence: CVRLHESCLGQQVPCCDP<u>A</u>ATCYCRFFNAFCYCR (SEQ ID NO:4). This sequence is

equivalent to the first 34 amino acids of MARP, but contain a Cys to Ala substitution at position 19 (indicated as "A") to avoid having a non-bridged thiol. The additional terminal residue (R) facilitates solid state synthesis. This mini-AGRP was shown to specifically bind an antagonize melanocortin receptors.

In addition, the mini-ARGPs can be modified to improve binding specificity, and/or to alter activity (*e.g.* agonist, antagonist, compeitive inhibitor, *etc.*). Such modifications include, but are not limited to changing the sequence to increase binding affinity, to increase the level of antagonism and to increase stability of the molecule. In particularly preferred embodiments, the amino acid substitutions are made in the RFF residues (residues 25-27 of SEQ ID NO:3) and/or in the N-terminal loop (*e.g.* one or more of residues 2-7 and/or residues 9-14 of SEQ ID NO:3).

In particularly preferred embodiments, mini-ARGPs are represented by formula I:

$$CX^1X^2X^3X^4X^5X^6CX^7X^8X^9X^{10}X^{11}X^{12}CCDPX^{13}ATCYCX^{14}X^{15}X^{16}NAFCYCR_n \quad I$$

where X^1, X^2, X^3, X^4, X^5, X^6, X^7, X^8, X^9, X^{10}, X^{11}, X^{12}, X^{13}, X^{14}, X^{15}, and X^{16} are independently selected amino acids (including natural, synthetic, or modified amino acids); and n is zero or one. In certain embodiments, in each of the varied domains, one or more of the native residues can be preserved. Thus, for example, $X^1X^2X^3X^4X^5X^6$ includes, but is not limited to $VX^2X^3X^4X^5X^6$, $X^1RX^3X^4X^5X^6$, $X^1X^2LX^4X^5X^6$, $X^1X^2X^3HX^5X^6$, $X^1X^2X^3X^4X^5S$, $VRX^3X^4X^5X^6$, $VX^2LX^4X^5X^6$, $VX^2X^3HX^5X^6$, $VX^2X^3X^4EX^6$, $VX^2X^3X^4X^5S$, $X^1RLX^4X^5X^6$, $X^1RX^3HX^5X^6$, $X^1RX^3X^4EX^6$, $X^1RX^3X^4X^5S$, $X^1X^2LHX^5X^6$, $X^1X^2LX^4X^5X^6$, $X^1X^2LX^4EX^6$, $X^1X^2LX^4X^5S$, $X^1X^2X^3HEX^6$, $X^1X^2X^3HX^5S$, $X^1X^2X^3X^4ES$, $VRLX^4X^5X^6$, $VX^2LHX^5X^6$, VRLHES and the like. Similar permutations are available for $X^7X^8X^9X^{10}X^{11}X^{12}$ (*e.g.* LGQQVP, $LX^8X^9X^{10}X^{11}X^{12}$, $X^7GX^9X^{10}X^{11}X^{12}$, $X^7X^8QX^{10}X^{11}X^{12}$, $X^7X^8X^9QX^{11}X^{12}$, $X^7X^8X^9X^{10}VX^{12}$, $X^7X^8X^9X^{10}X^{11}P$, $LGX^9X^{10}X^{11}X^{12}$, $LX^8QX^{10}X^{11}X^{12}$, $LX^8X^9QX^{11}X^{12}$, $LX^8X^9X^{10}VX^{12}$, $LX^8X^9X^{10}X^{11}P$, $LGQX^{10}X^{11}X^{12}$, and the like). Similarly, the "RFF" domain can be fully mutated or can retain one or more of the native residues. Thus, for example, $X^{14}X^{15}X^{16}$ includes RFF, $R^{15}X^{15}X^{16}$, $X^{14}FX^{16}$, $X^{14}X^{15}F$, RFX^{16}, $RX^{15}F$, $X^{15}FF$. In certain preferred embodiments, X^{13} is not cysteine.

The mini-AGRPs form a class of melanocortin receptor binding and/or modulating agents and thus find utility as modulators (*e.g.* upregulators, downregulators, competitive inhibitors) of melanocortin receptors, particular MC3r and/or MC4r. The compounds can be used individually or combined into a library suitable for screening for members having particular activities, binding affinities, and the like.

Certain mini-AGRPs are good potential therapeutics for the treatment of eating disorders and obesity, or can be used as therapeutic lead compounds for the development of therapeutics (*e.g.* as models for peptidomimetics, *etc.*). Where the compounds are used as therapetics, they can be administered to a patient (human or non-human mammal) in need thereof, to increase eating and fat deposition.

III. Preparation of mini-AGRP's and mini-AGRP libraries.

In certain embodiments, this invention provides libraries of mini-AGRPs. The libraries typically comprise a plurality of

Preferred libraries comprise at least 20, preferably at least 50, more preferably at least 100, and most preferably at least 10,000, 50,000, 100,000, or even at least 1,000,000 different members.

Using the mini-AGRP sequence information provided herein, such libraries can be routinely prepared using methods well known to those of skill in the art. Such methods include, but are not limited to "traditional" chemical syntheses methods, light-directed chemical syntheses, and recombinant expression.

Solid phase peptide synthesis in which the C-terminal amino acid of the sequence is attached to an insoluble support followed by sequential addition of the remaining amino acids in the sequence is the preferred method for preparing the peptide compounds of the present invention. Techniques for solid phase synthesis are well known t those of skill in the art (*see, e.g.*, Barany and Merrifield, *Solid-Phase Peptide Synthesis*; pp. 3-284 in The Peptides: Analysis, Synthesis, Biology. Vol. 2: Special Methods in Peptide Synthesis, Part A., Merrifield, et al. J. Am. Chem. Soc. 85, 2149-2156 (1963), and Gross and Meienhofer, eds. Academic press, N.Y., 1980 and Stewart et al., Solid Phase Peptide Synthesis, 2nd ed. Pierce Chem. Co., Rockford, Ill. (1984)). Solid phase synthesis is most easily accomplished with commercially available peptide synthesizers utilizing FMOC or TBOC chemistry.

In particularly preferred embodiments, peptide synthesis is performed using Fmoc synthesis. For example, AGRP C-terminal portion (MARP) can be readily synthesized using techniques described in Yang, et al. *supra*. Each desired peptide can be individually synthesized. Where large numbers of different peptides are desired (*e.g.* for a library) combinatorial synthesis methods are available. Combinatorial peptide synthesis can be performed according to the methods of Furka *et al.*, (1991) *Int. J. Pept. Protein Res.* 37 487-493. At the desired positions that are to be altered the synthesis resins are separated coupled with the desired amino acid(s) and then pooled for the next coupling reaction. Such methods are easily accomplished using multiple peptide synthesizers.

After synthesis, the proteins are purified if necessary (*e.g.* via HPCL), re-folded and disulfide bonds are formed. Formation of intrapeptide disulfide bonds is often achieved by oxidation of the free thiol or sulfur-protected precursors under varying reaction conditions (*see, e.g.,* Andreu *et al.* (1994) Pages 91-169 In *Peptide Synthesis Protocols;* Pennington, M. W., Bunn, B. M., Ed.; Humana Press: New Jersey; Moroder *et al.* (1996) *Biopolymers* 40: 207-234; Annis and Barany (1997) *Meth. Enzymol.*, 289: 198-221; Tam *et al.* (1991) *J. Am. Chem. Soc.* 111:, 6657-6662; Munson and Barany (1993) *J. Am. Chem. Soc. 115:* 10203-10216; Shik (1993) *J. Org. Chem.* 58: 3003-3008; Annis and Barany (1998) *J. Am. Chem. Soc.*, 120: 7226-7238; Shi and Rabenstein (1999) *J. Org. Chem.* 64: 4590-4595). Suitable oxidants include, but are not limited to iodine, thallium(III) trifluroacetate (Fujii *et al.*(1987) *Chem. Pharm. Bull.*, 35: 2339-2347), and the like.

Alternatively, recombinant expression methods can be used to produce the peptides of this invention. In this approach, a nucleic acid encoding the desired polypeptide (and optionally a purification tag, *e.g.* His_6) is provided in an appropirate vector. A cell (*e.g. E. coli*, SF-3 cell, *etc.*) is transfected with the nucleic acid and, under appropriate conditions, transcribes and translates the desired protein. The protein is then recovered, re-folded, if necessary and cross-linked using standard methods well known to those of skill in the art.

Molecular cloning techniques to achieve these ends are known in the art. A wide variety of cloning and in vitro amplification methods are suitable for the construction of recombinant nucleic acids. Examples of these techniques and instructions sufficient to direct persons of skill through many cloning exercises are found, *e.g.*, in Berger and Kimmel, (1989) *Guide to Molecular Cloning Techniques, Methods in Enzymology volume* 152 Academic Press, Inc., San Diego, CA; Sambrook *et al.* (1989) *Molecular Cloning - A*

Laboratory Manual (2nd ed.) Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor Press, NY; and Ausubel *et al.* (1994) *Current Protocols in Molecular Biology*, Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc.,

IV. Use of molecular models to design small molecules that bind the melanocortin receptor ligand binding site.

One aspect of the invention involves methods for identifying and designing small molecules that bind to the ligand binding site using atomic models of MARP. In particular, the invention provides methodsof identifying a compound that modulates ligand binding to a melanocortin receptor. The method involves modeling test compounds that mimic or match the 3D conformation of MARP and therefore are expected to fit spatially into a melanocortin receptor ligand binding site of interest, using an atomic structural model of a melanocortin receptor binding region or portion thereof, preferably comprising the MARP receptor binding region or portion thereof. The test compounds can fit spatially into the ligand binding site of interest based upon a geometric fit of its three-dimensional structure or based upon the spatial arrangement of atoms presenting specific chemical properties such as charge and hydrophobicity. The test compounds are then screened in an assay, such as a biological assay, characterized by binding of a test compound to a melanocortin receptor ligand binding site, and identifying a test compound that modulates ligand binding to the melanocortin receptor. Details of the atomic structural model are described in detail herein.

The atomic coordinates of MARP and its receptor binding region, provided herein, can be used for modeling to identify other compounds or fragments that bind melanocortin receptors. By "modeling" is intended quantitative and qualitative analysis of molecular structure/function based on atomic structural information and receptor-ligand agonists/antagonists interaction models. This includes conventional numeric-based molecular dynamic and energy minimization models, interactive computer graphic models, modified molecular mechanics models, distance geometry and other structure-based constraint models. Modeling is preferably performed using a computer and may be further optimized using known methods. By "fits spatially" is intended that the three-dimensional structure of a compound is accommodated geometrically by a cavity or pocket of a melanocortin receptor ligand binding site or by a pattern of charge or hydrophobicity.

Compounds of particular interest fit spatially and preferentially into the ligand binding site. By "fits spatially and preferentially" is intended that a compound possesses a three-dimensional structure and conformation for selectively interacting with a melanocortin receptor ligand binding site. Compounds that fit spatially and preferentially into the ligand binding site interact with amino acid residues forming the ligand binding site. More specifically, these compounds mimic or match the MARP receptor binding region. The present invention also includes a method for identifying a compound capable of selectively modulating ligand binding to different melanocortin receptors. The method comprises the steps of modeling test compounds that fit spatially and preferentially into the ligand binding site of a melanocortin receptor of interest using an atomic structural model of a MARP, screening the test compounds in a biological assay for melanocortin receptor activity characterized by preferential binding of a test compound to the ligand binding site of a melanocortin receptor, and identifying a test compound that selectively modulates the activity of a melanocortin receptor. Such receptor-specific compounds are selected that exploit differences between the ligand binding sites of one type of receptor versus a second type of receptor.

The invention also is applicable to generating new compounds that distinguish melanocortin receptor isoforms. This can facilitate generation of either tissue-specific or function-specific compounds.

The receptor-specific compounds of the invention preferably interact with conformationally constrained residues of the ligand binding site that are conserved among one type of receptor compared to a second type of receptor. "Conformationally constrained" is intended to refer to the three-dimensional structure of a chemical or moiety thereof having certain rotations about its bonds fixed by various local geometric and physical-chemical constraints. Conformationally constrained structural features of a ligand binding site include residues that have their natural flexible conformations fixed by various geometric and physical-chemical constraints, such as local backbone, local side chain, and topological constraints. These types of constraints are exploited to restrict positioning of atoms involved in receptor-ligand recognition and binding.

As described in the Examples, residues 25, 26 and 27 of the MARP receptor binding region are shown to be critical for activity. These three residues, along with the overall structure and composition of the N-terminus and central loop, appear to be necessary for optimal biological activity. Accordingly, modification to these residues can be exploited

in the identification and design of compounds that modulate ligand binding to one melanocortin receptor compared to another.

For modeling, docking algorithms and computer programs that employ them can be used to identify compounds that match or mimic the MARP receptor binding region. For example, docking programs can be used to predict how a small molecule of interest can interact with the melanocortin receptor ligand binding site. Fragment-based docking also can be used in building molecules *de novo* inside the ligand binding site, by placing chemical fragments that complement the site to optimize intermolecular interactions. The techniques can be used to optimize the geometry of the binding interactions. This design approach has been made possible by identification of the receptor binding region structure thus, the principles of molecular recognition can now be used to design a compound which matches the structure of this region. Compounds that structurally match or mimic the MARP receptor binding region serve as a starting point for an iterative design, synthesis and test cycle in which new compounds are selected and optimized for desired properties including affinity, efficacy, and selectivity. For example, the compounds can be subjected to addition modification, such as replacement and/or addition of R-group substituents of a core structure identified for a particular class of binding compounds, modeling and/or activity screening if desired, and then subjected to additional rounds of testing.

Computationally small molecule databases can be screened for chemical entities or compounds that can bind in whole, or in part, to a melanocortin receptor ligand binding site of interest. In this screening, the quality of fit of such entities or compounds to the binding site may be judged either by shape complementarity (DesJalais et al., *J. Med. Chem.* (1988) *31*:722-729) or by estimated interaction energy (Meng et al., *J. Comp. Chem.* (1992) *13*:505-524). The molecule databases include any virtual or physical database, such as electronic and physical compound library databases, and are preferably used in developing compounds that modulate ligand binding.

Compounds can be designed intelligently by exploiting available structural and functional information by gaining an understanding of the quantitative structure-activity relationship (QSAR), using that understanding to design new compound libraries, particularly focused libraries having chemical diversity of one or more particular groups of a core structure, and incorporating any structural data into that iterative design process. For example, one skilled in the art may use one of several methods to screen chemical entities or fragments to compare them to the 3D structure of the AGRP C-terminus and thus, their

ability to associate with the ligand binding site of a melanocortin receptor of interest. This process may begin by visual inspection of, for example, the receptor binding region on the computer screen. Selected fragments or chemical entities may then be positioned into all or part of the region. Docking may be accomplished using software such as Quanta and Sybyl, followed by energy minimization and molecular dynamics with standard molecular mechanics force-fields, such as CHARMM and AMBER.

Residues comprising a receptor binding region can be modeled to look for energetic contributions and interaction with the bound chemical entity. For example, a compound or fragment can be designed to contain hydrophobic groups that interact with hydrophobic residues of the ligand binding site.

Specialized computer programs may also assist in the process of selecting chemical entity fragments or whole compounds. These include: GRID (Goodford, *J. Med. Chem.* (1985) *28*:849-857; available from Oxford University, Oxford, UK); MCSS (Miranker et al., *Proteins: Structure, Function and Genetics*, (1991) *11*:29-34; available from Molecular Simulations, Burlington, MA); AUTODOCK (Goodsell et al., *Proteins: Structure, Function and Genetics* (1990) *8*:195-202; available from Scripps Research Institute, La Jolla, CA); and DOCK (Kuntz et al, *J. Mol. Biol.* (1982) *161*:269-288; available from University of California, San Francisco, CA).

Additional commercially available computer databases for small molecular compounds include Cambridge Structural Database and Fine Chemical Database (Rusinko, *Chem. Des. Auto. News* (1993) *8*:44-47).

Once suitable chemical entities or fragments have been selected, they can be assembled into a single compound. Assembly may be proceeded by visual inspection of the relationship of the fragments to each other on the three-dimensional image displayed on a computer screen in relation to the structure coordinates of a melanocortin receptor. This can be followed by manual model building using software such as Quanta or Sybyl.

Useful programs to aid one of skill in the art in connecting the individual chemical entities or fragments include: CAVEAT (Bartlett et al., "CAVEAT: A Program to Facilitate the Structure-Derived Design of Biologically Active Molecules", In: *Molecular Recognition in Chemical and Biological Problems*", Special Pub., *Royal Chem. Soc.* (1989) *78*:182-196; CAVEAT is available from the University of California, Berkeley, CA); 3D Database systems such as MACCS-3D (MDL Information Systems, San Leandro, CA;

reviewed in Martin, *J. Med. Chem.* (1992) *35*:2145-2154); and HOOK (available from Molecular Simulations, Burlington, MA).

In addition to building a compound in a step-wise fashion, one fragment or chemical entity at a time as described above, compounds that bind to a ligand binding site of interest also may be designed as a whole or *de novo* using some portion(s) of the AGRP C-terminus, which is a molecule known to bind to the site. These methods include: LUDI (Bohm, *J. Comp. Aid. Molec. Design* (1992) *6*:61-78; LUDI is available from Biosym Technologies, San Diego, CA); LEGEND (Nishibata et al., *Tetrahedron* (1991) *47*:8985; LEGEND is available from Molecular Simulations, Burlington, MA); and LeapFrog (available from Tripos Associates, St. Louis, MO).

Other molecular modeling techniques may also be employed in accordance with this invention. See, for example, Cohen et al., *J. Med. Chem.* (1990) *33*:883-894); Navia et al., *Curr. Opin. Struct. Biol.* (1992) 2:202-210). For example, where the structures of test compounds are known, a model of the test compound may be superimposed over the model of the structure of the invention. Numerous methods and techniques are known in the art for performing this step, any of which may be used. See, for example, Farmer, "*Drug Design*," Ariens, E.J., ed., 10:119-143 (Academic Press, New York, 1980); U.S. Patent No. 5,331,573; U.S. Patent No. 5,500,807; Verlinde, *Structure*, (1994) 2:577-587); and Kuntz et al., *Science,* (1992) *257*:1078-1082). The model building techniques and computer evaluation systems described herein are not a limitation on the present invention.

Using these computer modeling systems a large number of compounds may be quickly and easily examined and expensive and lengthy biochemical testing avoided. Moreover, the need for actual synthesis of many compounds can be substantially reduced and/or effectively eliminated.

V. AGRP peptidomimetics.

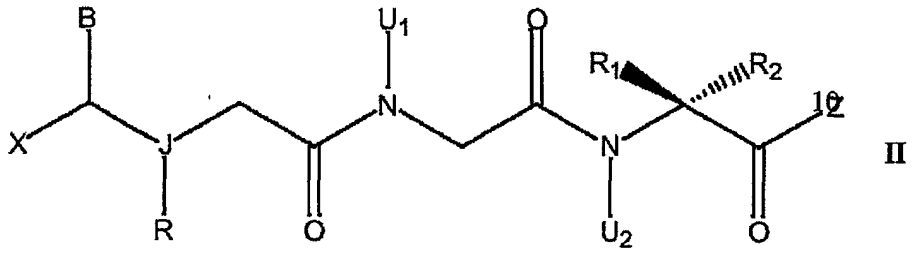
Using the three-dimensional model of AGRP provided herein, a class of non-peptide melanocortin receptor ligands (peptidomimetics) were designed. The subject ligands are low molecular weight compounds that structurally mimic the AGRP active loop backbone. The subject non-peptide compounds find use in a variety of different applications, including the modulation of melanocortin receptor mediated physiological processes.

By ligands of melanocortin receptors (MCR) is meant that the subject non-peptide compounds bind to melanocortin receptors. In many embodiments, the subject compounds preferentially bind to the following melanocortin receptors: MC3R, as described in U.S. Patent No. 5,837,521, the disclosure of which is herein incorporated by reference; and MC4R, as described in U.S. Patent No. 5,703,220, the disclosure of which is herein incorporated by reference. As the subject non-peptide compounds are MCR ligands, the subject compounds bind to an MCR with an affinity corresponding to a K_d of about 50 mM or lower, preferably of about 5 mM or lower, more preferably of about 500 μM or lower, and most preferably about 50 μM, 5 μM, or 1 μM or lower. In many embodiments, the affinity of the subject non-peptide compounds for an MCR, usually either MC3r or MC4r, ranges from about ranges from about 100 μM to 0.1 pM, and more usually about 10 μM to 0.1 nM As the subject compounds are non-peptide compounds, they are protease resistant. By protease resistant is meant that the subject compounds are resistant to proteolytic cleavage, at least by the proteases described in Miller *et al.* (1994), *Bioorg Med Chem Let* 4: 2657-2662. The subject non-peptide compounds are low molecular weight compounds. By low molecular weight is meant that the subject compounds have a molecular weight that ranges from about 200 to 1100 g/mol, usually from about 300 to 900 g/mol and more usually from about 400 to 800 g/mol.

A feature of the subject non-peptide compounds is that they structurally mimic the active loop 3-D conformation when bound by the receptor. By active loop is meant residues 111-116 or Arg-Phe-Phe-Asn-Ala-Phe (SEQ ID NO:__) of the Agouti Related Protein. More specifically, the subject non-peptide compounds are characterized by substantially structurally mimicking the backbone phi angle of amino acid 113 in AGRP, *i.e.* Phe113 phi = -55.4°, and the U_1-U_2 interatomic distance (see structure below). As the subject compounds substantially structurally mimic the active loop, in 9 of 10 lowest energy structures calculated with distance geometry the following requirements should be met.

The phi angle mimicking amino acid 113 of AGRP, any deviation from the above angles should not exceed about -90° to -10° or 10° to 90°, usually about -85° to -20° or 20° to 85°, and more usually about -80° to -30° or 30° to 80° in phi space. The interatomic U_1-U_2 distance should not exceed about 6.5 Å, usually about 5.7 Å, and more usually about about 5.5 Å.

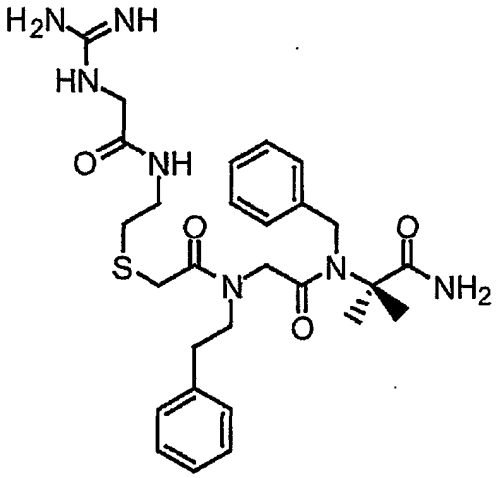
In many preferred embodiments, the subject non-peptide ligands have the formula of Formula II:



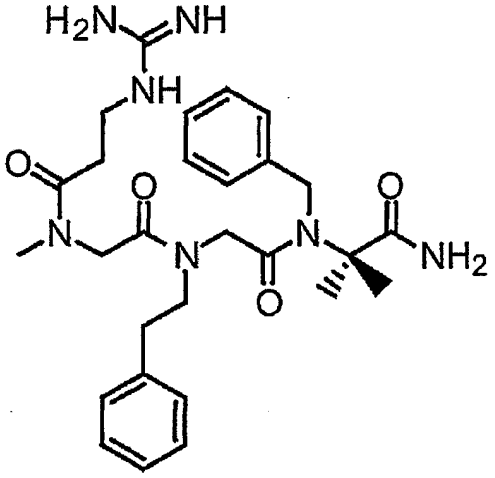
II

where: each B, U_1, U_2, R, R_1 and R_2 is independently selected from the group consisting of: hydrogen; alkyl; derivatized alkyl, *e.g.* halo alkyl, alkoxyalkyl, heteroalkyl, etc.; cycloalkyl; derivatized cycloalkyl, *e.g.* halocycloalkyl, aloxycycloalkyl, heterocycloalkyl; aryl; arylalkyl; heteroaryl; or heteroarylalkyl; J is carbon, nitrogen, silicon, or sulfur; X is hydrogen, carbon, nitrogen, oxygen, silicon, or sulfur; Z is a continuing peptide bond; hydroxyl; amide of the form $-NH_2$-, -NH-(n) or -N-(n,n'), where n or n' can be any alkyl, alkenyl, alkynyl, cycloalkyl, aryl, arylalkyl, heteroaryl or heteroarylalkyl, or derivatized form thereof; or ester of the form -O-(y) where y can be any alkyl, alkenyl, alkynyl, cycloalkyl, aryl, arylalkyl, heteroaryl or heteroarylalkyl, or derivatized form thereof. In certain prefered embodiments, B comprises a heteroalkyl moiety, where in certain preferred embodiments the heteroalkyl moiety comprises a guanidino moiety. In certain preferred embodiments, each U is a cycloalkyl, preferably arylalkyl, and more preferably methylbenzyl. In many preferred embodiments, the subject compounds are compounds in which B, R, R_1 and R_2 are as described above.

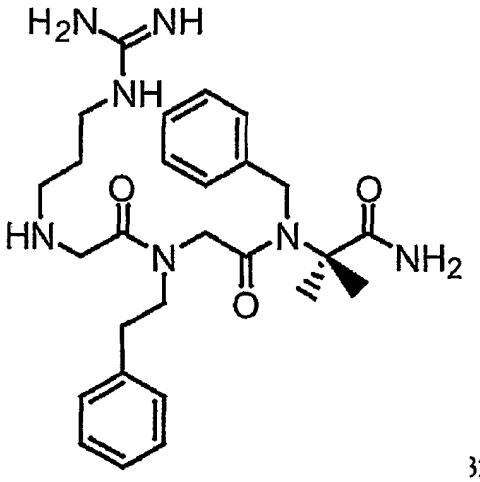
Specific non-peptide compounds of interest include the compounds of Formulas III, IV, V, and VI, shown below:



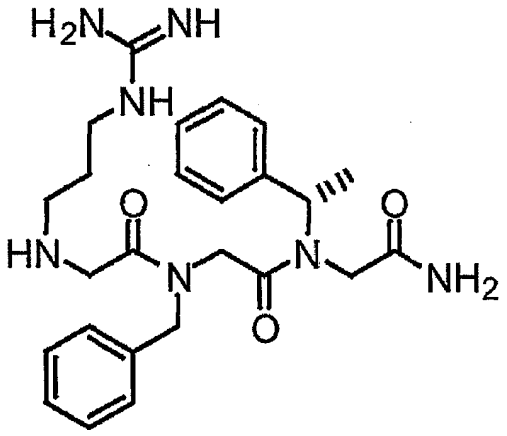
III



IV



V



VI.

The subject compounds may be synthesized using any convenient protocol. In one representative protocol, the peptoid portion of the subject molecules is synthesized using a submonomer approach, where a representative submonomer synthesis protocol that may be adapted to synthesize the subject compounds is described in U.S. Patent Nos. 5,977,301 and 5,831,005; the disclosures of which are herein incorporated by reference. Following production of the peptoid portion, the terminal "B" group as described above is added to the terminus of the peptoid portion of the compound. A representative sheme for preparation of the subject compounds is provided in the Experimental Section, *infra*.

VII. Uses of melanocortin receptor ligands.

A) Use as melanocortin receptor probes.

The melanocortin receptor ligands of this invention are useful in a wide variety of contexts. Because the ligands specifically bind the melanocortin receptor, they can be used as probes to specifically detect/localize melanocortin receptors (*in vivo, in vitro*, in cell culture, *etc.*). Thus, the ligands can be used to detect the presence or absence or to

quantify the expression level of a melanocortin receptor. In such applications, the receptor ligands are preferably labeled with a detectable label.

Detectable labels suitable for use in the present invention include any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Useful labels in the present invention include, but are not limited to biotin for staining with labeled streptavidin conjugate, magnetic beads (*e.g.*, DynabeadsTM), fluorescent dyes (*e.g.*, fluorescein, texas red, rhodamine), fluorescent proteins (*e.g.*, green fluorescent protein (GFP), red fluorescent protein (RFP), and the like), radiolabels (*e.g.*, ^{3}H, ^{125}I, ^{35}S, ^{14}C, or ^{32}P), enzymes (*e.g.*, horse radish peroxidase, alkaline phosphatase and others commonly used in an ELISA), chemiluminescent labels (*e.g.* luciferins), and colorimetric labels such as colloidal gold (*e.g.*, gold particles in the 40 -80 nm diameter size range scatter green light with high efficiency) or colored glass or plastic (*e.g.*, polystyrene, polypropylene, latex, etc.) beads. Other suitable labels include spin labels, *e.g.*, provided by reporter molecules with an unpaired electron spin which can be detected by electron spin resonance (ESR) spectroscopy. Exemplary spin labels include organic free radicals, transitional metal complexes, particularly vanadium, copper, iron, and manganese, and the like.

It will be recognized that fluorescent labels are not to be limited to single species organic molecules, but include inorganic molecules, multi-molecular mixtures of organic and/or inorganic molecules, crystals, heteropolymers, and the like. Thus, for example, CdSe-CdS core-shell nanocrystals enclosed in a silica shell can be easily derivatized for coupling to a biological molecule (Bruchez et al. (1998) Science, 281: 2013-2016). Similarly, highly fluorescent quantum dots (zinc sulfide-capped cadmium selenide) have been covalently coupled to biomolecules for use in ultrasensitive biological detection (Warren and Nie (1998) Science, 281: 2016-2018).

Patents teaching the use of labels include U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241.

The label may be added to the melanocortin receptor ligand prior to, or after the ligand is contacted to the sample. So called "direct labels" are detectable labels that are directly attached to or incorporated into the ligand prior application of the ligand to the sample. In contrast, so called "indirect labels" are joined to the bound ligand after it has contacted the sample. Often, the indirect label becomes attached to a binding moiety present on the ligand before it is contacted to the sample. Thus, for example, the ligand

may be biotinylated before it is used in the assay. After the ligand is bound to melanocortin receptor(s) in the sample, an avidin-conjugated fluorophore will bind the biotin on the ligand thereby providing a label that is easily detected.

The label is attached to the ligand directly or through a linker moiety. In certain embodiments, the label can be conveniently attached to an amino or carboxyl terminus of the ligand or to the R group of any amino acid(s) comprising the ligand as long as it does not interfere with specific binding of the ligand.

The label is detected using a method appropriate to the nature of the label. Thus, for example, enzymatic labels are detected by providing the appropriate substrate and reaction conditions for the enzyme and detecting loss of substrate or increase of reaction product. Radioactive labels are detected, *e.g.* via scintillography. Fluorescent labels and/or colorimetric labels are detected using optical methods (*e.g.* fluorometry, image analysis, etc).

B) Use in altering melanocortin receptor activity.

In certain preferred embodiments, the melanocortin receptor ligands of this invention are used to alter (modulate) melanocortin receptor activity. The ligand can act as a simple competitive inhibitor blocking access to the receptor by its native ligand. Alternatively the ligands of this invention can act as receptor agonists or antagonists.

The melanocortin receptor can be modulated *in vivo*, or *in vitro*. Of particular interest is use of such compounds in a method of modulating melanocortin receptor activity in a mammal by administering to a mammal in need thereof a sufficient amount of a compound that fits spatially and preferentially into a ligand binding site of a melanocortin receptor of interest. By "modulating" is intended increasing or decreasing activity of a melanocortin receptor.

The subject compounds (*e.g.* mini-AGRPs and/or non-peptide ligands) find use in a variety of different applications, and are particularly suited for use in modulating MC3R and/or MC4R mediated physiological processess. As such, the subject compounds find use in modulating feeding behaviour, and treating disease conditions associated with feeding behaviour. For example, MCR agonists or AGRP binding antagonists of the subject methods find use inhibiting appetite, where such compounds find use in the treatment of disease conditions associated with excessive caloric intake, *e.g.* obesity and the like. Conversely, MCR antagonists of the subject invention find use in treating disease

conditions where increasing appetite is attractive, *e.g.* anorexia, cachexia and the like. Other disease conditions in which the subject compounds may find use include those described in: WO 99/21517; WO 98/10068; WO 99/54358; WO 99/31508; WO 99/43709; WO 99/55679; and WO 99/57148.

Other representative uses for the subject ligands, *e.g.* screening assays for MCR ligands, etc., are also disclosed in these listed patent applications, the disclosures of which are herein incorporated by reference). Labeled, *i.e.* radio, fluorescent, biotin, antigen, etc., non-peptide ligands of melanocortin receptors find use as biological tracers for receptor identification *in vivo*.

The compounds of the subject invention find use in a variety of different hosts. Generally such hosts are "mammals" or "mammalian," where these terms are used broadly to describe organisms which are within the class mammalia, including the orders carnivore *(e.g.*, dogs and cats), rodentia (*e.g.*, mice, guinea pigs, and rats), and primates (*e.g.*, humans, chimpanzees, and monkeys). In many embodiments, the hosts will be humans.

The compouds of this invention are also useful as "lead compounds" for the development of therapeutics. For example, pre-clinical candidate compounds can be tested in appropriate animal models in order to measure efficacy, absorption, pharmacokinetics and toxicity following standard techniques known in the art. Compounds exhibiting desired properties are then tested in clinical trials for use in treatment of various melanocortin receptor-based disorders. These include feeding disorders, including wasting syndromes, obesity, and other disorders related to hypothalamic control of feeding. A wasting syndrome is an illness characterized by significant weight loss accompanied by other indicia of poor health, including poor appetite, gut disorder, or increased metabolic rate. Wasting syndromes include, but are not limited to, the wasting syndrome afflicting some patients diagnosed with Acquired Immune Deficiency Syndrome (AIDS) and various cancers. As methods of treating other symptoms of diseases such as AIDS progress, the incidence of wasting syndrome as the cause of death increases. Improved prophylaxis and treatment for HIV wasting syndrome is required (Kravick, et al., *Arch. Intern. Med.* 157:2069-2073, 1997). Anorexia and cachexia are well-known results of cancer that contribute to morbidity and mortality (Simons, et al, *Cancer* 82:553-560, 1998; Andrassy & Chwals, *Nutrition* 14:124-129, 1998). The reasons for the significant weight loss are multiple and may be directly related to the tumor, such as increased metabolic rate, but also include decreased

intake due to poor appetite or gut involvement. Further, excessive leptin-like signaling may contribute to the pathogenesis of wasting illness (Schwartz, et al., *Pro. Nutr. Soc.* 56:785-791, 1997).

Accordingly, one aspect of the invention pertains to a method of treating a disease state in mammals that is alleviated by treatment with a polypeptide having an amino acid sequence: $CX^1X^2X^3X^4X^5X^6CX^7X^8X^9X^{10}X^{11}X^{12}CCDPX^{13}ATCYCX^{14}X^{15}X^{16}N$ AFC YCR_n (SEQ ID NO:__), wherein X^1, X^2, X^3, X^4, X^5, X^6, X^7, X^8, X^9, X^{10}, X^{11}, X^{12}, X^{13}, X^{14}, X^{15}, and n is 0 or 1, which method comprises administering to a mammal in need of such a treatment a therapeutically effective amount of the polypeptide, which can be administered, by way of illustration and not limitation, in a liquid formulations or a solid formulations, such as in the form of a pharmaceutically acceptable salt thereof. In one preferred embodiment, the polypeptide has the amino acid sequence: CVRLHESCLGQQVPCC DPAATCYCRFFNAFCYC (SEQ ID NO:3). In certain embodiments, such a disease state can be a wasting syndrome.

There are many other uses and advantages provided by the present invention. For example, the methods and compositions described herein are useful for identifying peptides, peptidomimetics or small natural or synthetic organic molecules that modulate melanocortin receptor activity. The compounds are useful in treating melanocortin receptor-based disorders. Methods and compositions of the invention also find use in characterizing structure/function relationships of natural and synthetic ligand compounds

VIII. Screening of peptidomimetics and/or mini-AGRPs.

A) Binding and activity assays.

The compounds of this invention (*e.g.* mini-AGRPs or peptidomimetics) or libraries thereof can be screened to identify those having a particular receptor specificity, and/or avidity, and/or activity (*e.g.* agonist, antagonist, *etc.*). Such screening methods can readily identify "lead compounds" displaying the desired properties. Once lead compounds are identified, a variety of homologs and analogs can be prepared, *e.g.* to facilitate an understanding of the relationship between chemical structure and biological or functional activity. These studies define structure activity relationships which are then used to design drugs with improved potency, selectivity and pharmacokinetic properties. Combinatorial chemistry can also be used to rapidly generate a variety of structures for lead optimization.

In preferred embodiments, such assays include binding assays and/or activity assays. Binding assays typically measure the ability of the ligand(s) of interest to bind a melanocortin receptor (*e.g.* MC3r, MC4r, *etc.*). The assays can be qualitative (positive or negative) or quantitative, *e.g.* provide a measure of binding affinity.

Suitable binding assays are well known to those of skill in the art. In a preferred embodiment, the binding assay is a competitive assay that measures the ability of the test composition to displace/compete a reference ligand from a melanocortin receptor. In one preferred approach specific binding of ligands of this invention to cells expressing the MC-3 receptor is determined by competition experiments using labeled (*e.g.* ^{125}I-labeled) Nle^4-D-Phe^7-α-MSH (NDP-MSH), as described in Tatro *et al.* (1990) *Cancer Res.* 50: 1237-1242).

In various embodiments, screening can be *in vitro* and/or *in vivo*. Certain preferred assays include cell-free competition assays and cell culture based assays.

Activity assays measure the ability of the compounds of this invention to activate (agonize) or inhibit (antagonize) activity at one or more melanocortin receptors or to block or augment the activity of known antagonists or antagonists. Activity assays for melanocortin receptors are well known to those of skill in the art.

Preferred assays measure, directly, or indirrectly, melanocortin receptor induced changes in intracellular cAMP concentrations. One such assay measures the ability of cAMP to displace (8-^{3}H) cAMP from a high affinity cAMP binding protein (see Gilman (1979) *Proc. Natl. Acad. Sci., USA,* 67: 305-312). Briefly, test cells are exposed to the ligands to be screened. Following treatment, the cells are washed twice with phosphate buffered saline and intracellular cAMP extracted by lysing the cells with 1 mL of 60% ethanol. The assay is then run as described by Gilman, *supra.*).

Another convenient assay system is described in U.S. patent 6,100,048. This patent discloses recombinant expression constructs comprising nucleic acid encoding mammalian melanocortin receptors, and mammalian cells into which the recombinant expression constructs have been introduced that express functional mammalian melanocortin receptors. A panel is provided of such transformed mammalian cells expressing melanocortin receptors for screening compounds for receptor agonist and antagonist activity. One typical panel includes MC1r, MC2r, MC3r, and MC4r. In various embodiments, each of the cells of the panel of mammalian cells expressing mammalian

melanocortin receptors further comprises a recombinant expression construct encoding a cyclic AMP responsive element (CRE) transcription factor binding site that is operatively linked to a nucleic acid sequence encoding a protein capable of producing a detectable metabolite. Expression of the protein that produces the detectable metabolite is dependent on binding of the test compound to the melanocortin receptor expressed by each cell in the panel and the intracellular production of cAMP as a result. Detection of the metabolite thereby provides a measure of the agonist or antagonist activity of the ligand(s) in question.

In a preferred embodiment, compounds of the invention bind to a melanocortin receptor ligand binding site with greater affinity than the native cellular ligand proteins. Preferred compounds show at least a 1.25 fold greater affinity, preferably at least a 1.5 fold greater affinity, more preferably at least a 2 fold greater affinity, and most preferably at least a 5 fold or 10 fold greater affinity than the native cellular ligand proteins.

Preferred compounds can additionally or alternatively, show a different receptor specificity, *e.g.* specificity for MC4r rather than specificity for MC3r and MC4r, *etc.* The compounds selected can have agonist and/or antagonistic properties. The compounds also include those that exhibit new properties with varying mixtures of agonist and antagonist activities, depending on the effects of altering ligand binding in the context of different activities of melanocortin receptors which are mediated by proteins other than ligands, and which interact with the receptors at locations other than the ligand binding site. The compounds also include those, which through their binding to receptor locations that are conformationally sensitive to ligand binding, have allosteric effects on the receptor by stabilizing or destabilizing the ligand-bound conformation of the receptor, or by directly inducing the same, similar, or different conformational changes induced in the receptor by ligand binding.

The assays described herein are meant to be illustrative and not limiting. Using the teaching provided herein, other suitable assays will be apparent to those of skill in the art.

In certain preferred embodiments, the compounds that s

B) High throughput screening

The activity or binding assays of this invention are also amenable to "high-throughput" modalities. As described above preferred ligands of this invention bind to melanocortin receptors and thereby modulate receptor activity. Preferred assays detect MC

receptor binding and/or activity. High throughput assays for the presence, absence, or quantification of particular products are well known to those of skill in the art. Similarly, binding assays are similarly well known. Thus, for example, U.S. Patent 5,559,410 and 5,585,639 discloses high throughput binding assays.

In addition, high throughput screening systems are commercially available (see, *e.g.*, Zymark Corp., Hopkinton, MA; Air Technical Industries, Mentor, OH; Beckman Instruments, Inc. Fullerton, CA; Precision Systems, Inc., Natick, MA, etc.). These systems typically automate entire procedures including all sample and reagent pipetting, liquid dispensing, timed incubations, and final readings of the microplate in detector(s) appropriate for the assay. These configurable systems provide high throughput and rapid start up as well as a high degree of flexibility and customization. The manufacturers of such systems provide detailed protocols the various high throughput. Thus, for example, Zymark Corp. provides technical bulletins describing screening systems for detecting the modulation of gene transcription, ligand binding, and the like.

It is noted that HTS can be efficiently accomplished by screening combinations of ligands in a single assay. Thus, for example, a 680 well microtitre plate could contain 100 different ligands per well providing 68,000 different ligands per plate. Those wells that show a positive score for a particular assay are then sequentially subsampled to identify the particular ligand(s) providing the positive signal.

C) Databases of melanocortin receptor modulators.

In certain embodiments, the agents (ligands) that score positively in the assays described herein (*e.g.* show an ability to bind and/or modulate a melanocortin receptor) can be entered into a database of putative and/or actual modulators of Socs2 expression. Alternatively or additionally, a specificity fingerprint of a putative or acctual modulator can be entered into the database.

The term database refers to a means for recording and retrieving information. In preferred embodiments, the database also provides means for sorting and/or searching the stored information. The database can comprise any convenient media including, but not limited to, paper systems, card systems, mechanical systems, electronic systems, optical systems, magnetic systems or combinations thereof. Preferred databases include electronic (*e.g.* computer-based) databases. Computer systems for use in storage and manipulation of databases are well known to those of skill in the art and include, but are not limited to

"personal computer systems", mainframe systems, distributed nodes on an inter- or intra-net, data or databases stored in specialized hardware (*e.g.* in microchips), and the like.

IX. Methods for identifying receptor binding regions of melanocortin receptor agonist and antagonists.

The invention also includes compositions and methods for identifying receptor binding regions of melanocortin receptor agonist and antagonists, along with ligand binding sites of melanocortin receptors. The methods involve examining the surface of a polypeptide of interest to identify residues that modulate ligand binding. The residues can be identified by homology to the receptor binding region of MARP described herein. A preferred method is alignment with the residues of any polypeptide corresponding to (*i.e.*, the same as or equivalent to) residues 1-18 of the N-terminal loop (residues 1-18 of SEQ ID NO:2), residues 19-34 of the central loop (residues 19-34 of SEQ ID NO:2) and residues 35-46 of the C-terminal loop (residues 35-46 of SEQ ID NO:2). Overlays and superpositioning with a three-dimensional model of the MARP receptor binding region, or a portion thereof that contains a receptor binding region, also can be used for this purpose. For example, melanocortin receptor agonists and antagonists identifiable by homology alignment include naturally occurring compounds or compounds structurally related to such naturally occurring compounds found in humans, along with synthetic compounds.

Alignment and/or modeling also can be used as a guide for the placement of mutations on the receptor binding region surface to characterize the nature of the ligand binding site on melanocortin receptors in the context of a cell. To destroy the ligand binding interaction, preferred mutations are to charged residues (*e.g.*, Arg, Lys, or Glu) on the basis that bulky, surface charged residues might disrupt ligand binding, yet preserve the overall ligand structure and solubility. Mutants can be tested for ligand binding as well as the relative change in strength of the binding interaction. Ligand-dependent ligand interaction assays also can be tested for this purpose, such as those described herein.

Compounds that bind to the ligand binding site of melanocortin receptors can be identified by computational modeling and/or screening. For example, ligand agonists or antagonists can be identified by providing atomic coordinates comprising the MARP receptor binding region or portion thereof to a computerized modeling system, modeling them, and identifying compounds that mimic or match the receptor binding region and thus would be expected to fit spatially into the ligand binding site. By a "portion thereof" is

intended the atomic coordinates corresponding to a sufficient number of residues or their atoms of the receptor binding region that interact with a melanocortin receptor capable of binding the region. As another example, an atomic structural model utilized for computational modeling and/or screening of compounds that mimic or match the receptor binding region and thus would be expected to fit spatially into the ligand binding site, may include a portion of atomic coordinates of amino acid residues corresponding to the region composed of residues 24-31 of the central loop (residues 24-31 of SEQ ID NO:2), or their structural and functional equivalents. An atomic model can also be designed that includes residues 19-34 of the central loop (residues 19-34 of SEQ ID NO:2), and a model can further be designed that includes residues 19-34 of the central loop (residues 19-34 of SEQ ID NO:2) and some or all of residues 1-18 of the N-terminal loop (residues 1-18 of SEQ ID NO:2). Thus, for example, the atomic coordinates provided to the modeling system can contain atoms of MARP, all or part of the receptor binding region or a subset of atoms useful in the modeling and design of compounds that mimic or match the receptor binding region.

X. Pharmaceutical Preparations

Also provided are pharmaceutical preparations of the subject non-peptide compounds. The subject compounds can be incorporated into a variety of formulations for therapeutic administration. More particularly, the compounds of the present invention can be formulated into pharmaceutical compositions by combination with appropriate, pharmaceutically acceptable carriers or diluents, and may be formulated into preparations in solid, semi-solid, liquid or gaseous forms, such as tablets, capsules, powders, granules, ointments, solutions, suppositories, injections, inhalants and aerosols. The formulations may be designed for administration via a number of different routes, including oral, buccal, rectal, parenteral, intraperitoneal, intradermal, transdermal, intracheal, etc., administration.

In pharmaceutical dosage forms, the compounds may be administered in the form of their pharmaceutically acceptable salts, or they may also be used alone or in appropriate association, as well as in combination, with other pharmaceutically active compounds. The following methods and excipients are merely exemplary and are in no way limiting.

For oral preparations, the compounds can be used alone or in combination with appropriate additives to make tablets, powders, granules or capsules, for example, with

conventional additives, such as lactose, mannitol, corn starch or potato starch; with binders, such as crystalline cellulose, cellulose derivatives, acacia, corn starch or gelatins; with disintegrators, such as corn starch, potato starch or sodium carboxymethylcellulose; with lubricants, such as talc or magnesium stearate; and if desired, with diluents, buffering agents, moistening agents, preservatives and flavoring agents.

The compounds can be formulated into preparations for injection by dissolving, suspending or emulsifying them in an aqueous or nonaqueous solvent, such as vegetable or other similar oils, synthetic aliphatic acid glycerides, esters of higher aliphatic acids or propylene glycol; and if desired, with conventional additives such as solubilizers, isotonic agents, suspending agents, emulsifying agents, stabilizers and preservatives.

The compounds can be utilized in aerosol formulation to be administered via inhalation. The compounds of the present invention can be formulated into pressurized acceptable propellants such as dichlorodifluoromethane, propane, nitrogen and the like.

Furthermore, the compounds can be made into suppositories by mixing with a variety of bases such as emulsifying bases or water-soluble bases. The compounds of the present invention can be administered rectally via a suppository. The suppository can include vehicles such as cocoa butter, carbowaxes and polyethylene glycols, which melt at body temperature, yet are solidified at room temperature.

Unlike typical peptide formulations, the peptides of this invention comprising D-form amino acids can be administered, even orally, without protection against proteolysis by stomach acid, etc. Nevertheless, in certain embodiments, peptide delivery can be enhanced by the use of protective excipients. This is typically accomplished either by complexing the polypeptide with a composition to render it resistant to acidic and enzymatic hydrolysis or by packaging the polypeptide in an appropriately resistant carrier such as a liposome. Means of protecting polypeptides for oral delivery are well known in the art (see, *e.g.*, U.S. Patent 5,391,377 describing lipid compositions for oral delivery of therapeutic agents).

Where the ligands of this invention are polypeptides for oral administration, it is desirable to protect the polypeptide from digestion. This is readily accomplished by a variety of encapsulation technologies. One preferred encapsulation system is a "sustained-release system".

Such sustained release systems are well known to those of skill in the art and can often maintain elevated serum half-life. In one preferred embodiment, the ProLease

biodegradable microsphere delivery system for proteins and peptides (Tracy (1998) *Biotechnol. Prog.* 14: 108; Johnson et al. (1996) *Nature Med.* 2: 795; Herbert et al. (1998), *Pharmaceut. Res.* 15, 357) a dry powder composed of biodegradable polymeric microspheres containing the protein in a polymer matrix that can be compounded as a dry formulation with or without other agents.

The ProLease microsphere fabrication process was specifically designed to achieve a high protein encapsulation efficiency while maintaining protein integrity. The process consists of (i) preparation of freeze-dried protein particles from bulk protein by spray freeze-drying the drug solution with stabilizing excipients, (ii) preparation of a drug-polymer suspension followed by sonication or homogenization to reduce the drug particle size, (iii) production of frozen drug-polymer microspheres by atomization into liquid nitrogen, (iv) extraction of the polymer solvent with ethanol, and (v) filtration and vacuum drying to produce the final dry-powder product. The resulting powder contains the solid form of the protein, which is homogeneously and rigidly dispersed within porous polymer particles. The polymer most commonly used in the process, poly(lactide-co-glycolide) (PLG), is both biocompatible and biodegradable.

Encapsulation can be achieved at low temperatures (*e.g.*, -40°C). During encapsulation, the protein is maintained in the solid state in the absence of water, thus minimizing water-induced conformational mobility of the protein, preventing protein degradation reactions that include water as a reactant, and avoiding organic-aqueous interfaces where proteins may undergo denaturation. A preferred process uses solvents in which most proteins are insoluble, thus yielding high encapsulation efficiencies (*e.g.*, greater than 95%).

Unit dosage forms for oral or rectal administration such as syrups, elixirs, and suspensions may be provided wherein each dosage unit, for example, teaspoonful, tablespoonful, tablet or suppository, contains a predetermined amount of the composition containing one or more inhibitors. Similarly, unit dosage forms for injection or intravenous administration may comprise the inhibitor(s) in a composition as a solution in sterile water, normal saline or another pharmaceutically acceptable carrier.

The term "unit dosage form," as used herein, refers to physically discrete units suitable as unitary dosages for human and animal subjects, each unit containing a predetermined quantity of compounds of the present invention calculated in an amount sufficient to produce the desired effect in association with a pharmaceutically acceptable

diluent, carrier or vehicle. The specifications for the novel unit dosage forms of the present invention depend on the particular compound employed and the effect to be achieved, and the pharmacodynamics associated with each compound in the host.

The pharmaceutically acceptable excipients, such as vehicles, adjuvants, carriers or diluents, are readily available to the public. Moreover, pharmaceutically acceptable auxiliary substances, such as pH adjusting and buffering agents, tonicity adjusting agents, stabilizers, wetting agents and the like, are readily available to the public.

In therapeutic applications, the compositions of this invention are administered to a patient suffering from one or more symptoms of a wasting syndromes, obesity, and other disorders related to hypothalamic control of feeding. Such conditions include, but are not limited to wasting syndrome accompanying AIDS and various cancers. The compounds are administered in an amount sufficient to cure or at least partially prevent or arrest one or more symptoms of the the disease and/or its complications. An amount adequate to accomplish this is defined as a "therapeutically effective dose." Amounts effective for this use will depend upon the severity of the disease and the general state of the patient's health. Single or multiple administrations of the compositions may be administered depending on the dosage and frequency as required and tolerated by the patient. In any event, the composition should provide a sufficient quantity of the active agents of the formulations of this invention to effectively treat (ameliorate one or more symptoms) the patient.

The concentration of ligand (polypeptide or peptiodmimetic) can vary widely, and will be selected primarily based on fluid volumes, viscosities, body weight and the like in accordance with the particular mode of administration selected and the patient's needs. Concentrations, however, will typically be selected to provide dosages ranging from about 0.1 mg/kg/day to about 1000 mg/kg/day, preferably from about 1 mg/kg/day to about 100 mg/kg/day, more preferably from about 5 mg/kg/day to about 50 mg/kg/day. It will be appreciated that such dosages may be varied to optimize a therapeutic regimen in a particular subject or group of subjects.

XI. Kits.

In still another embodiment, this invention provides kits for practice of the methods described herein. In certain embodiments the kits comprise a container containing one or more of the melanocortin ligands of this invention. The ligands can be labeled or

unlabeled, and/or, optionally provided in a unit dosage form, and/or optionally, provided with or in a pharmacological acceptable excipient. The kits can optionally additionally include one or more detectable labels for labeling the ligand(s).

Certain preferred kits provide libraries of the ligands of this invention as described herein.

The kits can optionally include any reagents and/or apparatus to facilitate practice of the methods described herein. Such reagents and apparatus include, but are not limited to buffers, instrumentation, devices for administering the ligand(s) (*e.g.* syringes, *etc.*), microtiter plates, labeling reagents streptavidin or biotin conjugated substrates, PAGE gels, blotting membranes, reagents for detecting a signal, and the like.

In addition, the kits may include instructional materials containing directions (*i.e.*, protocols) for the practice of the methods of this invention. Preferred instructional materials provide protocols for utilizing the kit contents for modulating melanocortin receptor activity and/or for screening for particular melanocortin receptors, and/or for treating a disease or pathological state. While the instructional materials typically comprise written or printed materials they are not limited to such. Any medium capable of storing such instructions and communicating them to an end user is contemplated by this invention. Such media include, but are not limited to electronic storage media (*e.g.*, magnetic discs, tapes, cartridges, chips), optical media (*e.g.*, CD ROM), and the like. Such media may include addresses to internet sites that provide such instructional materials.

XII. Computer-based methods.

The invention also provides for the NMR structure of the human AGRP C-terminus, preferably embodied in a computer readable form. Synthesis of MARP and concentrated solutions adequate for NMR spectroscopy are described in the examples. After the NMR structure of MARP is determined, the structural information can be used in computational methods to design synthetic agonist and antagonist compounds for the melanocortin receptors, and further structure-activity relationships can be determined through routine testing using the assays described herein and known in the art. It is expected that the structure coordinates of the NMR structure of MARP, as provided in Tables 4 and 5, will be particularly useful for solving the NMR structure of other melanocortin receptor antagonists or agonists. The coordinates in Table 4 and Table 5 represent the minimized average of the coordinates of a family of NMR structures. The

minimized average was determined by (1) calculating an average from approximately 20 NMR structures found to meet the experimental NMR distance restraints and (2) subjecting this calculated average to a final round of energy minimization.

One method that may be employed for this purpose is molecular replacement. In this method, the unknown NMR structure, may be determined using the structure coordinates of this invention as provided in Table 4 and Table 5. The coordinates in Table 4 for the human AGRP C-terminus have been deposited with the Brookhaven National Laboratory Protein Data Bank, and have been assigned Brookhaven Protein Data Bank Accession Number 1qu8. The coordinates for Table 5 have been deposited with the Brookhaven National Laboratory Protein Data Bank, and have been assigned Brookhaven Protein Data Bank Accession Number 1HYK. This method will provide an accurate structural form for the unknown NMR structure more quickly and efficiently than attempting to determine such information *ab initio.*

One aspect of the invention is an NMR structure of the minimized agouti related protein, embodied in a computer readable media. Atomic coordinate information gleaned from the NMR structure of the invention is preferably stored and provided in the form of a machine-readable data storage medium. This medium contains information for constructing and/or manipulating an atomic model of a receptor binding region or portion thereof. For example, the machine readable data for the receptor binding region may comprise structure coordinates of amino acids corresponding to (*i.e.*, the same as or equivalent to) residues 1-18 of the N-terminal loop, (residues 1-18 of SEQ ID NO:2), and/or residues 19-34 of the central loop (residues 19-34 of SEQ ID NO:2) and/or residues 35-46 of the C-terminal loop (residues 35-46 of SEQ ID NO:2), or a homologue of the molecule or molecular complex comprising the region. The machine readable data for the receptor binding region may comprise structure coordinates of amino acids corresponding to residues 1-18 of the N-terminal loop, (residues 1-18 of SEQ ID NO:2) and residues 19-34 of the central loop (residues 19-34 of SEQ ID NO:2). The machine readable data may also comprise residues 24-31 of the central loop (residues 24-31 of SEQ ID NO:2) and a portion of the N-terminal loop, for example residues 15 to 18 (residues 15-18 of SEQ ID NO:2), residues 7 to 18 (residues 7-18 of SEQ ID NO:2), or residues 1 to 18 (residues 1-18 of SEQ ID NO:2). The homologues comprise a receptor binding region that has a root mean square deviation from the backbone atoms of the amino acids of not more than 2.54Å, preferably not more than 1.66 Å.

The machine-readable data storage medium can be used for interactive drug design and molecular replacement studies. For example, a data storage material is encoded with a first set of machine-readable data that can be combined with a second set of machine-readable data. For molecular replacement, the first set of data can comprise a Fourier transform of at least a portion of the structural coordinates of the AGRP C-terminus or portion thereof of interest, and the second data set comprises an X-ray diffraction pattern of a molecule such as the melanocortin receptor of interest. Using a machine programmed with instructions for using the first and second data sets a portion or all of the structure coordinates corresponding to the second data can be determined.

EXAMPLES

The following examples are offered to illustrate, but not to limit the claimed invention.

Example 1

Chemical synthesis and characterization of MARP

Abbreviations

$^3J_{HN\alpha}$	three bond αH-NH scalar coupling constant
AGRP/ART	Agouti Related Protein/Agouti Related Transcript
Conformational shift	experimental chemical shift–random coil chemical shift
DQF-COSY	two-dimensional double-quantum filtered correlation spectroscopy
HX	hydrogen-deuterium exchange
ICK	Inhibitor Cystine Knot
MARP	Minimized human Agouti Related Protein, residues 87-132 of human AGRP
MC3r/MC4r	Melanocortin Receptor ¾
MRE	Mean Residue Ellipticity
α-MSH	α-Melanocyte Stimulating Hormone
NDP-MSH	[Nle^4, D-Phe^7]α-MSH, a superpotent melanocortin agonist
nOe	melanocortin Overhauser enhancement
NOESY	two-dimensional melanocortin Overhauser spectroscopy
TOCSY	two-dimensional total correlation spectroscopy

Methods and Materials

A) Chemical protein synthesis:

N^{α}-Acetyl-MARP (SEQ ID NO:2) and N^{α}-acetyl-MARP(Arg25Ala) were synthesized, folded and purified to each give a protein containing five disulfide bonds, as reported in Yang, et al., *supra.*

B) CD experiments:

Circular dichroism ("CD") spectra were recorded at 25°C on an Aviv 60DS spectropolarimeter in a rectangular 1 mm path length cuvet for concentrations up to 60 μM. For concentrations higher than this a round cell with a 0.1 mm path length was used. All CD samples were 50 mM potassium phosphate, pH 4.25. Concentration dependence was ruled out in the range 20 μM-1 mM. Temperature dependence was determined for 5-85°C. The spectra are superimposable from 5 to 45°C.

C) NMR sample preparation:

The activity of MARP used for the NMR sample and that of a single mutant were assayed by measuring the inhibition of cAMP production in the presence of NDP-MSH (Shutter, et al., *supra*; Huszar, et al., *supra*) in HEK-293 cells transfected with human MC4r. Control experiments were performed with no MARP. The NMR samples were found to be native-like with complete activity. NMR samples contained approximately 1.9 mM MARP at pH 4.2 in 50 mM KH_2P0_4 buffer in 90% H_2O/10% D_2O. Additional samples for HX experiments were prepared by lyophilization of protonated samples followed by reconstitution in 700 μl D_2O.

D) NMR experiments:

^{1}H 2D NMR spectra were principally acquired at 15°C on a Varian 500 Unity Plus spectrometer using inverse probes. Initial NMR data were routinely acquired with a 6000 Hz spectral width, 4096 complex points in t_2, and 512 (TOCSY/DQF-COSY) or 700 (NOESY) increments in t_1. All spectra were processed using the MNMR package (Carlsberg Laboratory, Department of Chemistry, Denmark) and analyzed using XEASY (Bartels, et al., *J. Biomol. NMR* 5:1-10 (1995)), with chemical shifts referenced to 1.4-dioxime at 3.743 ppm. Sequential assignments of all backbone and >90% of side chain

protons were accomplished using standard methods (Wüthrich, K. "NMR of Proteins and Nucleic Acids" (John Wiley and Sons, New York, 1986); Redfield, C. in "NMR of Macromolecules: A Practical Approach, pp. 71-99 (Roberts, G.K.C., Ed., IRL Press at Oxford University Press, Oxford, 1993)) for 50 ms TOCSY, 150 ms NOESY and DQF-COSY data. Additional data sets were acquired at 25°C and 30°C to resolve ambiguities. Examination of the three Pro residues identified nOes consistent only with trans-Pro. The same experiments were repeated at 800 mHz.

Four additional peaks were identified in the αN region of the TOCSY spectrum, however, associated spin systems could not be identified and neither could nOes to the peaks in question.

NOESY data for distance restraints were collected at 15°C using the WET sequence (Smallcombe, et al., *J. Magn. Reson. Ser. A* 117:295-303 (1995)) for water suppression, 1.6 s recycle delay and a mixing time of 80 ms in both H_2O and D_2O. $^3J_{HN\alpha}$coupling constants at 25°C were determined by both linear least squares fitting of the antiphase doublets in a DQF-COSY and also using the INFIT (Szyperski, et al., *J. Magn. Reson.* 99:552-560 (1992)) module of XEASY with 150 ms NOESY data. These methods agreed to within ±0.5 Hz for all of the measured coupling constants. At 15°C larger intrinsic linewidths precluded accurate measurement of $^3J_{HN\alpha}$.

For amide exchange experiments, the magnet was preshimmed on a 21 residue peptide sample at pH 4 in D_2O/phosphate buffer. The first TOCSY experiment was begun 23 minutes after reconstituting the protonated sample in D_2O. Four TOCSY experiments identical to those described above, except for the number of t_1 increments, were acquired back to back at 15°C over a period of 24 hours. The first three consisted of 150 t_1 increments and the final experiment 300 increments. NOESY and DQF-COSY spectra were also acquired as described above.

E. Structure calculations:

Final structure calculations included the covalent connectivity of the published disulfide map (Bures, et al., *supra*), and were based on a total of 414 interproton distance constraints derived from the 80 ms 2D NOESY spectra and 34 backbone Φ dihedral angle constraints derived from coupling constant measurements, giving a total of 448 total restraints, or 9.7 restraints per residue. The distance restraints can be broken down into 228 intraresidue (backbone to side chain only), 129 sequential, 20 medium range ($1<_1 i$-

$j_1 \leq 5$) and 37 long range ($_1i$-$j_1 > 5$) restraints. These restraints were assigned as strong, medium or weak. The total numbers of restraints in each category were 95 strong, 246 medium, and 77 weak. All categories had a lower limit of 1.6Å, with upper limits of 2.8, 3.5 and 5 Å for the strong, medium and weak categories, respectively. Trial structures were generated using the simulated annealing protocol from CNS version 0.4a (anneal.inp) with SUM averaging for the nOe distances (Brunger, et al., *Acta Crystallogr. Sect. D Biol. Crystallogr.* 54:905-921 (1998); Nilges, et al., *FEBS Lett.* 239:129-136 (1988); Nilges, et al., *Protein Eng.* 2:27-38 (1988); Kuszewski, et al., *J. Magn. Reson. Ser. B* 112:79-81 (1996); Stein, et al., *J. Magn. Reson.* 124:154-164 (1997)). Twenty structures with no bond or nOe angle violations were used to represent the solution structure of MARP (see Table 1 for Root Mean Square Deviations, "RMSDs"). Structures were displayed using MOLMOL (Koradi, et al., *Science* 278:135-138 (1997)).

Results and Discussion.

The synthesis and biochemical characterization of MARP were previously reported in Yang, et al., *supra.* The N-terminal residue (Cys-1) of MARP corresponds to the first Cys (Cys-87) of the Cys-rich region in full length, 132 residue human AGRP. The material used for the MARP NMR sample showed native-like activity as measured by its ability to competitively inhibit NDP-MSH at MC4r, as has been shown in previous studies (Yang, et al., *supra*; Ollmann, et al., *Science* 278:135-138 (1997)). Previous mutational studies of agouti and AGRP showed residues Arg-25, Phe-26, Phe-27 (the RFF triplet, Tota, et al., *supra*) and Asp-17 (in agouti) to be determinants of receptor binding (Kiefer, et al., *Biochemistry* 36:2084-2090 (1997); Kiefer, et al., *Biochemistry* 37:991-997 (1998); Tota, et al., *supra*)).

This experiment measured inhibition of NDP-MSH stimulated cAMP generation in cells transfected with MC4r. A control experiment was conducted with no MARP present. The addition of MARP (5.0×10^{-6} M) demonstrated competitive inhibition of NDP-MSH. The addition of MARP with Arg-25 to Ala substitution (5.0×10^{-6} M) demonstrated loss of inhibition due to a mutation in the active loop. This experiment thus showed that the replacement of Arg-25 by Ala results in a complete loss of inhibitory activity.

The far-UV CD spectrum of MARP was measured in 50 mM phosphate buffer at pH 4.25 as a function of temperature with spectra shown every 5°C from 5°C to

30°C and also at 45°C. The spectrum was similar to that reported for a similar C-terminal fragment (residues 85-132) of AGRP (residues 85-132 of SEQ ID NO:1) reported by Rosenfeld, et al., *supra*, and is characterized by a negative maximum at approximately 198 nm and a slight negative maximum at 245 nm. There was little indication of canonical α-helix, however the spectrum did suggest some β character or turns. The near-UV CD spectrum, indicative of tertiary structure, showed a weak minimum at approximately 275 nm which could be attributed to the disulfide bonds and possibly restricted orientations of the side chains of the Tyr-23 and Tyr-32.

At 25°C between 20 μM and 1.0 mM there was no observable concentration dependence as measured by CD. Between 1 mM and 1.9 mM there were no concentration dependent changes in the NMR spectra (*i.e.* linewidths, chemical shifts, etc. all remain constant). The far-UV CD spectrum remained constant between 5°C and 45°C, a temperature range well beyond that of the present NMR experiments. Thus, by all indications, MARP existed as a monomer and did not exhibit temperature dependence under the conditions of the NMR experiments. The characteristics of the NMR spectra were indicative of a well folded protein with a single predominant conformer. $^3J_{HN\alpha}$ coupling constants and temperature coefficient also indicated a fully folded, non-random coil conformer as shown in Table 1 below, which shows the MARP coupling constants at 25°C and NH temperature coefficients in 50 min phosphate buffer at pH 4.2.

Table 1. $^3J_{HN\alpha}$ coupling constants and temperature coefficients.

Residue	$^3J_{NH\alpha}$ coupling constant (Hz)	NH temperature coefficient (ppb/°C)
Cys-l		6.93
Val-2	8.79	3.63
Arg-3	7.00	6.37
Leu-4	5.64	3.47
His-5	7.50	2.58
Glu-6	9.34	2.90
Ser-7		5.86
Cys-8	9.92	3.84
Leu-9	4.40	2.43
Gly-10		7.06
Gln-11	8.14	4.94
Gln-12	8.00	4.38
Val-13	9.03	5.56

Residue	$^3J_{NH\alpha}$ coupling constant (Hz)	NH temperature coefficient (ppb/°C)
Pro-14		0.00
Cys-15	4.33	6.74
Cys-16	5.42	3.10
Asp-17		4.81
Pro-18		0.00
Cys-19	8.81	3.84
Ala-20	9.64	3.23
Thr-21	9.36	4.04
Cys-22	5.76	7.56
Tyr-23		2.01
Cys-24		3.14
Arg-25	5.90	2.93
Phe-26	8.70	3.67
Phe-27	3.68	6.21
Asn-28	7.82	5.66
Ala-29	7.56	2.10
Phe-30	7.59	4.84
Cys-31	9.17	5.84
Tyr-32	9.49	4.43
Cys-33		1.13
Arg-34	9.34	2.74
Lys-35	7.25	8.80
Leu-36	7.74	6.43
Gly-37		7.00
Thr-38	8.07	2.07
Ala-39	4.48	7.67
Met-40	7.50	2.98
Asn-41	8.19	1.08
Pro-42		0.00
Cys-43	7.24	7.86
Ser-44	7.26	8.38
Arg-45	7.54	4.47
Thr-46		4.61

Example 2

Structural description of MARP

The minimized average NMR structure of MARP is shown in Figure 1. Consistent with the far-UV CD spectrum, MARP showed little evidence of helical or regular sheet secondary structure. The disulfide bonds (1-16, 8-22, 15-33, 19-43 and 24-31) appeared to form a scaffold upon which the structure was apportioned into three major loops, which are referred to as the N-terminal loop (residues 1-18), the central loop (residues 19-34) and the C-terminal loop (residues 35-46), and are indicated in Figure 1. RMSDs for the individual loops are reported in Table 2 below:

Table 2. Summary of MARP backbone and heavy atom RMSDs

Region (residues)	Backbone RMSD* (Å)	Heavy atom RMSD[a] (Å)
Global (1-46)	2.54	3.26
N- and active loops (1-34)	1.66	2.38
N-terminal loop (1-18)	1.31	2.03
Central loop (19-34)	1.51	2.22
Active loop (24-31)	0.69	1.53
C-terminal loop (35-46)	2.36	3.43

* Determined by fitting the family of 20 NMR structures to the minimized average structure

The N-terminal and central loops were much better defined both within the loops and with respect to each other than the C-terminal loop. The backbone RMSD for the entire protein (2.54Å) was of the same order as that of the C-terminal loop (2.36Å), while the backbone RMSD for residues 1-34 (1.66Å) was of the order of the individual N-terminal and central loops. To demonstrate limited backbone structure variability of the N-terminal and central loops, a superposition of 14 structures (selected for clarity) for residues 1-34 and the MARP minimized average structure (residues 1-46) is shown in Figure 2.

Four of the five disulfide bonds are located at the base of the structure where they appear to pinch together the bottoms of the loops to form the "core" of the protein (Figure 1). The exception is disulfide bond 24-31 which stabilizes the central loop. The central loop, residues 19-34, contains the RFF triplet determined to be critical for activity. This motif is situated within an even smaller, well defined loop bound by Cys-24 and Cys-31 which is referred to as the "active" loop. The side chain atoms of the RFF triplet residues are located at the surface of the protein as depicted in Figure 3. Recent

experiments further highlight the importance of this active loop. These studies demonstrate that short cyclic peptides corresponding to residues 24-31 of human AGRP do in fact antagonize MC3r and MC4r (Tota, et al., *supra*).

Inspection of the family of NMR structures and consideration of the observed HX revealed a structure for the central loop that is best described as an irregular hairpin with a well defined loop from Cys-24 to Cys-31 (RMSD 0.6Å, Figure 3) and a stem region which is both twisted around and curved along its *z*-axis (Figure 1). This characterization was supported by critical examination of the nOe, $^3J_{HN\alpha}$ and chemical shift data. As shown in Figure 3, the active loop is highly constrained with the RFF triplet side chains exposed to solvent. Arg-25 and Phe-27 point out into the solvent, while one face of the Phe-26 aromatic ring rests parallel against the surface of the protein. Though the active loop satisfies several of the determinants for an Ω-loop (Leszczynski, et al. (1986) *Science* 234:849-855), the side chain orientation of Arg-25 and Phe-27 precludes its definition as such since Ω-loop side chains generally pack within the loop of backbone atoms.

HX experiments demonstrated that the amide protons of residues Cys-8, Ala-20, Thr-1, Tyr-23, Tyr-2, Cys-3 and Arg-34 are protected from exchange with solvent. To explore whether these results were consistent with the average structure, the program DSSP (Kabsch, et al., *Biopolymers* 22:2577-2637 (1983)) was used to identify potential hydrogen bonds. DSSP identified the backbone amides of Ala-20, Thr-21, Tyr-23, and Arg-34 as potential hydrogen bond donors. In addition, solvent accessible surface area calculations showed that residues Cys-8 and Cys-33 were completely buried from solvent, though in the D_2O spectrum the αN crosspeaks of these two residues overlap, thus their individual protection from exchange is uncertain. Tyr-32 had only 8% solvent accessible surface area at the $C_\delta$protons.

The NMR structure (Table 4) gave a well resolved fold, however, as mentioned previously, canonical helices and β-sheets were not identified on the basis of nOes or other protocols including the chemical shift index (Wishart, et al., *Biochemistry* 31:1647-1651 (1992)) or $^3J_{HN\alpha}$ coupling constants (Wüthrich, K., *supra*).

The guidelines for these protocols assigned secondary structure on the basis of four or more consecutive residues with similar conformational shifts or $^3J_{HN\alpha}$. Helical structure was characterized by $^3J_{HN\alpha}<6$ Hz and negative conformational shifts and β-sheet by $^3J_{HN\alpha}>8$ Hz and positive conformational shifts. Even in the active loop and stem region

of the central loop, no regular secondary structure was identified by these criteria. However, the chemical shift index pointed towards a possible extended strand from residue 31 to residue 35. Table 3 sets forth the ^{1}H chemical shifts at 15°C, $^{3}J_{NH\alpha}$, at 250°C, and the NH temperature coefficients.

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Table 3. MARP ^{1}H chemical shifts at 15°C in 50 mm phosphate buffer at pH 4.2

Residue	NH (ppm)	H_α (ppm)	H_β (ppm)	Other (ppm)
Cys- 1	8.00	5.00	3.12, 2.81	
Val-2	8.92	4.18	1.94, 2.08	CH^{γ}_3 0.91, 0.80
Arg-3	8.91	4.01	1.67, 1.95	H^{γ}1.69, 1.82, H^{δ}3.24, 3.26, NH 7.31
Leu-4	8.13	3.72	1.34, 1.54	H^{γ}1.03, CH^{δ}_3 0.79
His-5	8.99	4.25	3.70, 1.42	H^{δ}7.22, $^{H\varepsilon}$8.51
Glu-6	8.04	4.66	2.16	H^{γ}2.24
Ser-7	8.63	4.68	3.84	
Cys-8	8.08	4.96	3.57, 3.13	
Leu-9	7.92	4.08	1.57, 1.44	H^{γ}1.44, $CH^{\delta}_3$0.82
Gly-10	8.78	4.07, 3.69		
Gln- 11	8.06	4.24	1.95	H^{γ} 2.28. $H^{\varepsilon 2}$ 6.86, 7.49
Gln- 12	8.68	4.27	2.20, 1.95	H^{γ} 2.30, 2.34, $H^{\varepsilon 2}$ 6.82, 7.45
Val- 13	7.48	4.47	2.05	CH^{γ}_3 0.79, 0.90
Pro-14	8.81	4.55	2.25, 1.99	H^{γ} 1.84, 1.99, H^{δ} 3.65, 3.77
Cys-15		4.92	3.35, 1.74	
Cys-16	9.58	4.22	2.62, 3.17	
Asp-17	8.19	4.78	2.63, 2.41	
Pro-18	8.88	4.50	2.35	H^{γ} 1.98, H^{δ} 3.89, 4.04
Cys-19		4.72	2.94, 3.47	
Ala-20	8.01	4.94	1.26	
Thr-21	8.76	4.58	4.00	CH^{γ}_3 1.16
Cys-22	8.94	4.58	2.94, 3.02	
Tyr-23	8.71	4.62	2.79	H^{δ} 6.93, H^{ε} 6.74
Cys-24	8.30	4.91	3.23, 2.60	
Arg-25	8.29	3.84	1.83, 1.55	H^{γ}1.56, 1.23, H^{δ} 3.06
Phe-26	7.91	4.74	2.80, 3.32	H^{δ} 7.26, H^{ε} 7.42
Phe-27	8.54	4.19	3.12, 3.02	H^{δ} 7.17, H^{ε} 7.33
Asn-28	8.48	4.20	2.39, 7.77	$H^{\delta 2}$ 6.66, 7.30
Ala-29	7.75	4.38	1.25	

Residue	NH (ppm)	H_α (ppm)	H_β (ppm)	Other (ppm)
Phe-30	8.38	4.21	3.32, 3.36	
Cys-31	8.34	5.63	2.59, 3.03	
Tyr-32	8.88	5.20	2.59, 2.81	H^δ 6.92, H^ε 6.66
Cys-33	8.19	4.96	3.21, 2.66	
Arg-34	9.43	4.75	1.83, 1.70	H^γ1.57, 1.69, H^δ 2.62, 2.88, NH 7.08
Lys-35	9.09	4.47	1.82, 1.68	H^γ1.25, 1.43, H^δ 1.64, H^ε 2.89
Leu-36	8.77	4.3640	1.63	H^γ1.52, $CH^\delta{}_3$ 0.82, 0.70
Gly-37	8.48	4.09, 4.01		
Thr-38	7.82	4.42	4.42	$CH^\gamma{}_3$ 1.21
Ala-39	8.53	4.16	1.42	
Met-40	8.06	4.40	1.95, 2.09	H^γ2.51, 2.62
Asn-41	7.74	5.00	2.62, 2.81	$H^{\delta 2}$ 7.67, 6.95
Pro-42	8.68	4.47	2.29	H^γ1.94, H^δ 3.61, 3.67
Cys-43		4.59	3.22, 3.13	
Ser-44	8.37	4.46	3.86	
Arg-45	8.26	4.43	1.93	H^γ1.66, H^δ 3.21, NH 7.25
Thr-46	7.86	4.15	4.23	$CH^\gamma{}_3$1.15

Table 4. MARP atomic coordinates in 3 dimensional space determine by NMR at 500 mHz.

ATOM	1	CA	CYS	1	-6.900	2.269	-8.250	1.00	4.62
ATOM	2	HA	CYS	1	-5.897	2.041	-7.920	1.00	4.59
ATOM	3	HB1	CYS	1	-7.160	4.143	-9.269	1.00	5.13
ATOM	4	HB2	CYS	1	-6.230	4.254	-7.780	1.00	4.63
ATOM	5	C	CYS	1	-7.900	1.623	-7.293	1.00	3.97
ATOM	6	O	CYS	1	-9.067	1.425	-7.636	1.00	4.16
ATOM	7	CB	CYS	1	-7.088	3.796	-8.249	1.00	4.78
ATOM	8	SG	CYS	1	-8.576	4.376	-7.358	1.00	5.12
ATOM	9	N	CYS	1	-7.069	1.766	-9.642	1.00	5.38
ATOM	10	HT1	CYS	1	-8.026	2.021	-9.958	1.00	5.84
ATOM	11	HT2	CYS	1	-6.942	0.734	-9.624	1.00	5.52
ATOM	12	HT3	CYS	1	-6.346	2.222	-10.234	1.00	5.55
ATOM	13	N	VAL	2	-7.445	1.356	-6.070	1.00	3.32
ATOM	14	HN	VAL	2	-6.517	1.593	-5.848	1.00	3.27
ATOM	15	CA	VAL	2	-8.315	0.801	-5.028	1.00	2.84
ATOM	16	HA	VAL	2	-9.187	0.372	-5.505	1.00	3.11
ATOM	17	CB	VAL	2	-7.624	-0.292	-4.151	1.00	2.58

ATOM	18	HB	VAL	2	-6.927	0.197	-3.490	1.00	2.54
ATOM	19	CG1	VAL	2	-8.637	-1.028	-3.287	1.00	2.26
ATOM	20	HG11	VAL	2	-9.533	-1.215	-3.860	1.00	2.67
ATOM	21	HG12	VAL	2	-8.879	-0.427	-2.423	1.00	2.47
ATOM	22	HG13	VAL	2	-8.214	-1.969	-2.962	1.00	2.24
ATOM	23	CG2	VAL	2	-6.851	-1.295	-5.000	1.00	3.27
ATOM	24	HG21	VAL	2	-6.240	-1.922	-4.350	1.00	3.49
ATOM	25	HG22	VAL	2	-6.214	-0.766	-5.692	1.00	3.65
ATOM	26	HG23	VAL	2	-7.544	-1.914	-5.548	1.00	3.65
ATOM	27	C	VAL	2	-8.750	1.967	-4.145	1.00	2.69
ATOM	28	O	VAL	2	-8.214	3.071	-4.285	1.00	3.12
ATOM	29	N	ARG	3	-9.694	1.759	-3.238	1.00	2.64
ATOM	30	HN	ARG	3	-10.100	0.873	-3.138	1.00	2.81
ATOM	31	CA	ARG	3	-10.120	2.851	-2.380	1.00	2.90
ATOM	32	HA	ARG	3	-10.214	3.738	-2.998	1.00	3.25
ATOM	33	CB	ARG	3	-11.466	2.539	-1.720	1.00	3.25
ATOM	34	HB1	ARG	3	-11.575	3.157	-0.841	1.00	3.44
ATOM	35	HB2	ARG	3	-11.477	1.501	-1.422	1.00	3.38
ATOM	36	CG	ARG	3	-12.662	2.785	-2.628	1.00	3.73
ATOM	37	HG1	ARG	3	-13.287	1.905	-2.628	1.00	4.00
ATOM	38	HG2	ARG	3	-12.307	2.976	-3.630	1.00	3.91
ATOM	39	CD	ARG	3	-13.486	3.978	-2.164	1.00	4.23
ATOM	40	HD1	ARG	3	-13.348	4.788	-2.865	1.00	4.60
ATOM	41	HD2	ARG	3	-13.136	4.285	-1.189	1.00	4.30
ATOM	42	NE	ARG	3	-14.913	3.662	-2.078	1.00	4.74
ATOM	43	HE	ARG	3	-15.185	2.745	-2.294	1.00	4.90
ATOM	44	CZ	ARG	3	-15.856	4.540	-1.726	1.00	5.32
ATOM	45	NH1	ARG	3	-15.532	5.796	-1.425	1.00	5.50
ATOM	46	HH11	ARG	3	-14.578	6.091	-1.459	1.00	5.28
ATOM	47	HH12	ARG	3	-16.246	6.446	-1.162	1.00	6.06
ATOM	48	NH2	ARG	3	-17.129	4.160	-1.677	1.00	6.01
ATOM	49	HH21	ARG	3	-17.379	3.219	-1.903	1.00	6.19
ATOM	50	HH22	ARG	3	-17.837	4.816	-1.413	1.00	6.50
ATOM	51	C	ARG	3	-9.053	3.103	-1.329	1.00	2.75
ATOM	52	O	ARG	3	-8.519	2.163	-0.738	1.00	2.50
ATOM	53	N	LEU	4	-8.725	4.372	-1.111	1.00	3.24
ATOM	54	HN	LEU	4	-9.167	5.082	-1.615	1.00	3.68
ATOM	55	CA	LEU	4	-7.708	4.726	-0.146	1.00	3.40
ATOM	56	HA	LEU	4	-6.847	4.143	-0.371	1.00	3.11

ATOM	57	CB	LEU	4	-7.351	6.196	-0.264	1.00	4.25
ATOM	58	HB1	LEU	4	-7.379	6.479	-1.304	1.00	4.60
ATOM	59	HB2	LEU	4	-6.353	6.339	0.110	1.00	4.61
ATOM	60	CG	LEU	4	-8.285	7.097	0.500	1.00	4.65
ATOM	61	HG	LEU	4	-8.344	6.741	1.509	1.00	4.56
ATOM	62	CD1	LEU	4	-7.773	8.520	0.517	1.00	5.17
ATOM	63	HD11	LEU	4	-7.232	8.714	-0.396	1.00	5.39
ATOM	64	HD12	LEU	4	-7.113	8.647	1.363	1.00	5.60
ATOM	65	HD13	LEU	4	-8.605	9.203	0.599	1.00	5.27
ATOM	66	CD2	LEU	4	-9.659	6.991	-0.103	1.00	5.22
ATOM	67	HD21	LEU	4	-10.392	7.382	0.584	1.00	5.44
ATOM	68	HD22	LEU	4	-9.857	5.945	-0.295	1.00	5.42
ATOM	69	HD23	LEU	4	-9.690	7.541	-1.030	1.00	5.58
ATOM	70	C	LEU	4	-8.186	4.418	1.274	1.00	3.37
ATOM	71	O	LEU	4	-9.386	4.241	1.507	1.00	3.67
ATOM	72	N	HIS	5	-7.244	4.346	2.217	1.00	3.48
ATOM	73	HN	HIS	5	-6.311	4.491	1.965	1.00	3.68
ATOM	74	CA	HIS	5	-7.564	4.044	3.618	1.00	3.77
ATOM	75	HA	HIS	5	-6.629	3.939	4.150	1.00	3.80
ATOM	76	CB	HIS	5	-8.361	5.193	4.240	1.00	4.60
ATOM	77	HB1	HIS	5	-8.651	4.923	5.245	1.00	4.82
ATOM	78	HB2	HIS	5	-9.248	5.373	3.650	1.00	4.71
ATOM	79	CG	HIS	5	-7.583	6.467	4.309	1.00	5.19
ATOM	80	ND1	HIS	5	-8.108	7.657	4.768	1.00	5.95
ATOM	81	HD1	HIS	5	-9.021	7.792	5.094	1.00	6.16
ATOM	82	CD2	HIS	5	-6.300	6.730	3.966	1.00	5.27
ATOM	83	HD2	HIS	5	-5.586	6.017	3.564	1.00	4.82
ATOM	84	CE1	HIS	5	-7.182	8.597	4.702	1.00	6.46
ATOM	85	HE1	HIS	5	-7.307	9.629	4.993	1.00	7.11
ATOM	86	NE2	HIS	5	-6.076	8.060	4.221	1.00	6.10
ATOM	87	HE2	HIS	5	-5.204	8.506	4.184	1.00	6.44
ATOM	88	C	HIS	5	-8.338	2.726	3.736	1.00	3.44
ATOM	89	O	HIS	5	-8.921	2.423	4.779	1.00	3.81
ATOM	90	N	GLU	6	-8.318	1.949	2.656	1.00	2.90
ATOM	91	HN	GLU	6	-7.824	2.247	1.869	1.00	2.77
ATOM	92	CA	GLU	6	-8.984	0.664	2.592	1.00	2.75
ATOM	93	HA	GLU	6	-9.613	0.554	3.462	1.00	3.21
ATOM	94	CB	GLU	6	-9.838	0.592	1.327	1.00	2.53
ATOM	95	HB1	GLU	6	-9.174	0.488	0.478	1.00	2.28

ATOM	96	HB2	GLU	6	-10.392	1.513	1.227	1.00	2.71
ATOM	97	CG	GLU	6	-10.825	-0.566	1.307	1.00	2.93
ATOM	98	HG1	GLU	6	-11.828	-0.167	1.356	1.00	3.17
ATOM	99	HG2	GLU	6	-10.645	-1.192	2.168	1.00	3.31
ATOM	100	CD	GLU	6	-10.701	-1.416	0.055	1.00	3.19
ATOM	101	OE1	GLU	6	-11.236	-1.006	-0.997	1.00	3.57
ATOM	102	OE2	GLU	6	-10.068	-2.491	0.127	1.00	3.55
ATOM	103	C	GLU	6	-7.955	-0.456	2.557	1.00	2.53
ATOM	104	O	GLU	6	-8.269	-1.602	2.876	1.00	2.98
ATOM	105	N	SER	7	-6.729	-0.115	2.130	1.00	2.02
ATOM	106	HN	SER	7	-6.557	0.801	1.870	1.00	1.87
ATOM	107	CA	SER	7	-5.656	-1.086	2.013	1.00	1.98
ATOM	108	HA	SER	7	-4.753	-0.563	1.693	1.00	1.74
ATOM	109	CB	SER	7	-5.409	-1.725	3.371	1.00	2.69
ATOM	110	HB1	SER	7	-5.970	-2.643	3.442	1.00	3.00
ATOM	111	HB2	SER	7	-5.742	-1.040	4.133	1.00	2.94
ATOM	112	OG	SER	7	-4.036	-2.005	3.572	1.00	3.30
ATOM	113	HG	SER	7	-3.939	-2.883	3.947	1.00	3.67
ATOM	114	C	SER	7	-6.066	-2.147	0.984	1.00	2.01
ATOM	115	O	SER	7	-7.207	-2.143	0.517	1.00	2.80
ATOM	116	N	CYS	8	-5.180	-3.074	0.643	1.00	1.78
ATOM	117	HN	CYS	8	-4.285	-3.074	1.044	1.00	1.85
ATOM	118	CA	CYS	8	-5.558	-4.119	-0.301	1.00	2.24
ATOM	119	HA	CYS	8	-6.476	-3.820	-0.745	1.00	2.23
ATOM	120	HB1	CYS	8	-3.606	-4.528	-1.025	1.00	2.70
ATOM	121	HB2	CYS	8	-4.503	-3.311	-1.949	1.00	2.24
ATOM	122	C	CYS	8	-5.763	-5.451	0.432	1.00	2.87
ATOM	123	O	CYS	8	-5.575	-6.522	-0.140	1.00	3.43
ATOM	124	CB	CYS	8	-4.568	-4.261	-1.425	1.00	2.52
ATOM	125	SG	CYS	8	-5.047	-5.510	-2.659	1.00	3.51
ATOM	126	N	LEU	9	-6.155	-5.353	1.714	1.00	3.11
ATOM	127	HN	LEU	9	-6.288	-4.468	2.091	1.00	3.01
ATOM	128	CA	LEU	9	-6.400	-6.509	2.578	1.00	3.82
ATOM	129	HA	LEU	9	-7.159	-7.116	2.111	1.00	4.03
ATOM	130	CB	LEU	9	-5.122	-7.346	2.756	1.00	4.45
ATOM	131	HB1	LEU	9	-5.161	-7.820	3.726	1.00	4.87
ATOM	132	HB2	LEU	9	-4.275	-6.676	2.739	1.00	4.44
ATOM	133	CG	LEU	9	-4.890	-8.438	1.701	1.00	4.92
ATOM	134	HG	LEU	9	-4.327	-8.018	0.881	1.00	4.96

ATOM	135	CD1	LEU	9	-4.073	-9.583	2.281	1.00	5.65
ATOM	136	HD11	LEU	9	-3.633	-9.273	3.217	1.00	5.93
ATOM	137	HD12	LEU	9	-3.291	-9.852	1.587	1.00	5.76
ATOM	138	HD13	LEU	9	-4.715	-10.435	2.449	1.00	6.14
ATOM	139	CD2	LEU	9	-6.214	-8.951	1.149	1.00	5.23
ATOM	140	HD21	LEU	9	-6.026	-9.607	0.313	1.00	5.47
ATOM	141	HD22	LEU	9	-6.812	-8.111	0.821	1.00	5.42
ATOM	142	HD23	LEU	9	-6.743	-9.490	1.921	1.00	5.45
ATOM	143	C	LEU	9	-6.919	-6.051	3.948	1.00	3.94
ATOM	144	O	LEU	9	-7.894	-6.604	4.462	1.00	4.53
ATOM	145	N	GLY	10	-6.267	-5.033	4.530	1.00	3.63
ATOM	146	HN	GLY	10	-5.501	-4.627	4.073	1.00	3.32
ATOM	147	CA	GLY	10	-6.681	-4.513	5.824	1.00	4.02
ATOM	148	HA1	GLY	10	-5.848	-4.575	6.507	1.00	4.32
ATOM	149	HA2	GLY	10	-7.490	-5.120	6.203	1.00	4.54
ATOM	150	C	GLY	10	-7.146	-3.066	5.745	1.00	3.77
ATOM	151	O	GLY	10	-8.151	-2.771	5.104	1.00	4.10
ATOM	152	N	GLN	11	-6.406	-2.169	6.402	1.00	3.61
ATOM	153	HN	GLN	11	-5.613	-2.477	6.886	1.00	3.76
ATOM	154	CA	GLN	11	-6.727	-0.736	6.407	1.00	3.67
ATOM	155	HA	GLN	11	-6.984	-0.452	5.397	1.00	3.70
ATOM	156	CB	GLN	11	-7.923	-0.451	7.325	1.00	4.51
ATOM	157	HB1	GLN	11	-7.690	0.399	7.949	1.00	4.89
ATOM	158	HB2	GLN	11	-8.094	-1.312	7.954	1.00	4.88
ATOM	159	CG	GLN	11	-9.210	-0.148	6.570	1.00	4.88
ATOM	160	HG1	GLN	11	-9.043	-0.320	5.517	1.00	5.09
ATOM	161	HG2	GLN	11	-9.467	0.889	6.727	1.00	4.85
ATOM	162	CD	GLN	11	-10.376	-1.008	7.023	1.00	5.60
ATOM	163	OE1	GLN	11	-10.540	-2.140	6.568	1.00	6.07
ATOM	164	NE2	GLN	11	-11.195	-0.474	7.924	1.00	6.05
ATOM	165	HE21	GLN	11	-11.006	0.433	8.244	1.00	5.94
ATOM	166	HE22	GLN	11	-11.958	-1.009	8.229	1.00	6.67
ATOM	167	C	GLN	11	-5.510	0.082	6.852	1.00	3.45
ATOM	168	O	GLN	11	-5.279	0.248	8.052	1.00	4.02
ATOM	169	N	GLN	12	-4.718	0.570	5.893	1.00	2.94
ATOM	170	HN	GLN	12	-4.930	0.397	4.955	1.00	2.77
ATOM	171	CA	GLN	12	-3.520	1.338	6.219	1.00	3.05
ATOM	172	HA	GLN	12	-3.399	1.297	7.292	1.00	3.57
ATOM	173	CB	GLN	12	-2.294	0.679	5.573	1.00	3.41

ATOM	174	HB1	GLN	12	-1.434	1.314	5.730	1.00	3.57
ATOM	175	HB2	GLN	12	-2.471	0.583	4.512	1.00	3.50
ATOM	176	CG	GLN	12	-1.976	-0.700	6.130	1.00	4.12
ATOM	177	HG1	GLN	12	-1.131	-1.105	5.591	1.00	4.31
ATOM	178	HG2	GLN	12	-2.834	-1.340	5.984	1.00	4.40
ATOM	179	CD	GLN	12	-1.639	-0.671	7.609	1.00	4.78
ATOM	180	OE1	GLN	12	-0.584	-0.178	8.007	1.00	5.27
ATOM	181	NE2	GLN	12	-2.538	-1.199	8.436	1.00	5.19
ATOM	182	HE21	GLN	12	-3.359	-1.574	8.052	1.00	5.11
ATOM	183	HE22	GLN	12	-2.342	-1.191	9.396	1.00	5.76
ATOM	184	C	GLN	12	-3.640	2.820	5.813	1.00	2.91
ATOM	185	O	GLN	12	-4.438	3.561	6.388	1.00	3.44
ATOM	186	N	VAL	13	-2.821	3.248	4.843	1.00	2.75
ATOM	187	HN	VAL	13	-2.199	2.625	4.444	1.00	2.86
ATOM	188	CA	VAL	13	-2.795	4.629	4.377	1.00	3.05
ATOM	189	HA	VAL	13	-3.226	5.246	5.153	1.00	3.57
ATOM	190	CB	VAL	13	-1.331	5.091	4.154	1.00	3.46
ATOM	191	HB	VAL	13	-1.291	6.137	4.363	1.00	3.85
ATOM	192	CG1	VAL	13	-0.380	4.402	5.127	1.00	3.80
ATOM	193	HG11	VAL	13	-0.395	3.337	4.956	1.00	3.78
ATOM	194	HG12	VAL	13	-0.691	4.607	6.140	1.00	4.15
ATOM	195	HG13	VAL	13	0.622	4.776	4.976	1.00	4.28
ATOM	196	CG2	VAL	13	-0.878	4.877	2.709	1.00	3.89
ATOM	197	HG21	VAL	13	-0.918	5.815	2.176	1.00	4.21
ATOM	198	HG22	VAL	13	-1.528	4.163	2.224	1.00	4.18
ATOM	199	HG23	VAL	13	0.135	4.503	2.700	1.00	4.14
ATOM	200	C	VAL	13	-3.615	4.813	3.090	1.00	3.04
ATOM	201	O	VAL	13	-4.211	3.857	2.589	1.00	2.95
ATOM	202	N	PRO	14	-3.671	6.053	2.539	1.00	3.66
ATOM	203	CA	PRO	14	-4.438	6.341	1.318	1.00	4.09
ATOM	204	HA	PRO	14	-5.448	5.975	1.401	1.00	4.39
ATOM	205	CB	PRO	14	-4.454	7.870	1.263	1.00	5.04
ATOM	206	HB1	PRO	14	-5.342	8.242	1.750	1.00	5.62
ATOM	207	HB2	PRO	14	-4.437	8.197	0.234	1.00	5.18
ATOM	208	CG	PRO	14	-3.224	8.289	1.986	1.00	5.32
ATOM	209	HG1	PRO	14	-3.368	9.268	2.418	1.00	5.91
ATOM	210	HG2	PRO	14	-2.384	8.296	1.308	1.00	5.77
ATOM	211	CD	PRO	14	-3.010	7.267	3.068	1.00	4.46
ATOM	212	HD1	PRO	14	-3.474	7.590	3.989	1.00	4.68

ATOM	213	HD2	PRO	14	-1.956	7.102	3.216	1.00	4.53
ATOM	214	C	PRO	14	-3.795	5.765	0.053	1.00	3.77
ATOM	215	O	PRO	14	-2.821	5.013	0.129	1.00	4.07
ATOM	216	N	CYS	15	-4.341	6.138	-1.114	1.00	3.60
ATOM	217	HN	CYS	15	-5.104	6.751	-1.110	1.00	3.75
ATOM	218	CA	CYS	15	-3.814	5.676	-2.394	1.00	3.70
ATOM	219	HA	CYS	15	-2.780	5.986	-2.427	1.00	3.90
ATOM	220	HB1	CYS	15	-4.871	3.825	-2.642	1.00	4.62
ATOM	221	HB2	CYS	15	-3.464	3.720	-1.587	1.00	4.34
ATOM	222	C	CYS	15	-4.533	6.333	-3.587	1.00	3.85
ATOM	223	O	CYS	15	-4.584	7.561	-3.661	1.00	4.33
ATOM	224	CB	CYS	15	-3.851	4.149	-2.500	1.00	4.05
ATOM	225	SG	CYS	15	-2.856	3.502	-3.887	1.00	4.02
ATOM	226	N	CYS	16	-5.057	5.523	-4.536	1.00	3.70
ATOM	227	HN	CYS	16	-4.985	4.556	-4.441	1.00	3.50
ATOM	228	CA	CYS	16	-5.710	6.043	-5.736	1.00	4.08
ATOM	229	HA	CYS	16	-5.636	5.275	-6.496	1.00	3.93
ATOM	230	HB1	CYS	16	-7.294	7.293	-4.999	1.00	4.46
ATOM	231	HB2	CYS	16	-7.597	5.564	-4.810	1.00	4.09
ATOM	232	C	CYS	16	-4.979	7.301	-6.249	1.00	4.59
ATOM	233	O	CYS	16	-5.610	8.273	-6.672	1.00	5.05
ATOM	234	CB	CYS	16	-7.196	6.327	-5.471	1.00	4.36
ATOM	235	SG	CYS	16	-8.216	6.341	-6.984	1.00	5.13
ATOM	236	N	ASP	17	-3.630	7.259	-6.197	1.00	4.60
ATOM	237	HN	ASP	17	-3.198	6.451	-5.847	1.00	4.30
ATOM	238	CA	ASP	17	-2.777	8.374	-6.639	1.00	5.13
ATOM	239	HA	ASP	17	-2.901	8.469	-7.708	1.00	5.59
ATOM	240	CB	ASP	17	-3.219	9.692	-5.975	1.00	5.62
ATOM	241	HB1	ASP	17	-3.503	9.496	-4.953	1.00	5.76
ATOM	242	HB2	ASP	17	-4.072	10.083	-6.508	1.00	5.74
ATOM	243	CG	ASP	17	-2.132	10.754	-5.977	1.00	6.17
ATOM	244	OD1	ASP	17	-1.748	11.210	-7.075	1.00	6.53
ATOM	245	OD2	ASP	17	-1.668	11.129	-4.880	1.00	6.56
ATOM	246	C	ASP	17	-1.283	8.093	-6.351	1.00	4.80
ATOM	247	O	ASP	17	-0.465	8.102	-7.271	1.00	4.56
ATOM	248	N	PRO	18	-0.908	7.839	-5.070	1.00	5.13
ATOM	249	CA	PRO	18	0.485	7.553	-4.669	1.00	5.06
ATOM	250	HA	PRO	18	1.165	8.301	-5.023	1.00	5.33
ATOM	251	CB	PRO	18	0.438	7.600	-3.127	1.00	5.85

ATOM	252	HB1	PRO	18	1.243	8.221	-2.761	1.00	6.32
ATOM	253	HB2	PRO	18	0.542	6.601	-2.729	1.00	5.79
ATOM	254	CG	PRO	18	-0.891	8.182	-2.786	1.00	6.39
ATOM	255	HG1	PRO	18	-0.814	9.257	-2.712	1.00	6.94
ATOM	256	HG2	PRO	18	-1.249	7.765	-1.857	1.00	6.75
ATOM	257	CD	PRO	18	-1.800	7.803	-3.912	1.00	5.92
ATOM	258	HD1	PRO	18	-2.598	8.519	-4.012	1.00	6.35
ATOM	259	HD2	PRO	18	-2.194	6.810	-3.762	1.00	5.99
ATOM	260	C	PRO	18	0.965	6.192	-5.162	1.00	4.35
ATOM	261	O	PRO	18	0.484	5.690	-6.181	1.00	4.45
ATOM	262	N	CYS	19	1.908	5.589	-4.438	1.00	4.00
ATOM	263	HN	CYS	19	2.257	6.027	-3.634	1.00	4.34
ATOM	264	CA	CYS	19	2.427	4.285	-4.822	1.00	3.50
ATOM	265	HA	CYS	19	2.416	4.237	-5.903	1.00	3.76
ATOM	266	HB1	CYS	19	4.354	5.069	-4.280	1.00	4.02
ATOM	267	HB2	CYS	19	4.412	3.492	-5.049	1.00	4.20
ATOM	268	C	CYS	19	1.501	3.201	-4.285	1.00	2.98
ATOM	269	O	CYS	19	0.949	2.444	-5.067	1.00	2.87
ATOM	270	CB	CYS	19	3.880	4.100	-4.333	1.00	3.68
ATOM	271	SG	CYS	19	4.070	3.305	-2.701	1.00	3.27
ATOM	272	N	ALA	20	1.301	3.192	-2.958	1.00	3.00
ATOM	273	HN	ALA	20	1.746	3.891	-2.427	1.00	3.43
ATOM	274	CA	ALA	20	0.381	2.259	-2.261	1.00	2.68
ATOM	275	HA	ALA	20	0.867	1.965	-1.352	1.00	2.43
ATOM	276	CB	ALA	20	-0.896	2.998	-1.882	1.00	2.88
ATOM	277	HB1	ALA	20	-1.747	2.351	-2.033	1.00	3.03
ATOM	278	HB2	ALA	20	-0.997	3.878	-2.499	1.00	3.21
ATOM	279	HB3	ALA	20	-0.847	3.293	-0.841	1.00	3.09
ATOM	280	C	ALA	20	0.077	0.972	-3.049	1.00	2.88
ATOM	281	O	ALA	20	-0.665	1.040	-3.996	1.00	3.63
ATOM	282	N	THR	21	0.639	-0.179	-2.606	1.00	2.52
ATOM	283	HN	THR	21	1.178	-0.127	-1.824	1.00	2.17
ATOM	284	CA	THR	21	0.492	-1.519	-3.247	1.00	3.01
ATOM	285	HA	THR	21	0.640	-1.411	-4.288	1.00	3.44
ATOM	286	CB	THR	21	1.615	-2.430	-2.712	1.00	3.51
ATOM	287	HB	THR	21	2.292	-1.827	-2.145	1.00	3.87
ATOM	288	OG1	THR	21	2.342	-3.018	-3.765	1.00	3.92
ATOM	289	HG1	THR	21	1.829	-3.731	-4.147	1.00	4.04
ATOM	290	CG2	THR	21	1.139	-3.534	-1.814	1.00	3.64

ATOM	291	HG21	THR	21	1.148	-4.469	-2.352	1.00	3.73
ATOM	292	HG22	THR	21	0.138	-3.302	-1.513	1.00	3.73
ATOM	293	HG23	THR	21	1.776	-3.606	-0.946	1.00	3.90
ATOM	294	C	THR	21	-0.893	-2.141	-3.040	1.00	2.82
ATOM	295	O	THR	21	-1.823	-1.429	-2.757	1.00	2.79
ATOM	296	N	CYS	22	-1.047	-3.453	-3.225	1.00	3.23
ATOM	297	HN	CYS	22	-0.284	-3.987	-3.488	1.00	3.75
ATOM	298	CA	CYS	22	-2.361	-4.091	-3.092	1.00	3.29
ATOM	299	HA	CYS	22	-2.941	-3.546	-2.350	1.00	2.73
ATOM	300	HB1	CYS	22	-2.576	-4.621	-5.160	1.00	4.32
ATOM	301	HB2	CYS	22	-3.066	-2.975	-4.783	1.00	4.25
ATOM	302	C	CYS	22	-2.268	-5.563	-2.656	1.00	3.83
ATOM	303	O	CYS	22	-2.573	-6.463	-3.444	1.00	4.63
ATOM	304	CB	CYS	22	-3.092	-3.999	-4.442	1.00	3.86
ATOM	305	SG	CYS	22	-4.841	-4.522	-4.418	1.00	3.79
ATOM	306	N	TYR	23	-1.888	-5.785	-1.377	1.00	3.64
ATOM	307	HN	TYR	23	-1.689	-5.024	-0.821	1.00	3.23
ATOM	308	CA	TYR	23	-1.799	-7.133	-0.783	1.00	4.31
ATOM	309	HA	TYR	23	-2.770	-7.368	-0.375	1.00	4.75
ATOM	310	CB	TYR	23	-1.483	-8.137	-1.875	1.00	4.99
ATOM	311	HB1	TYR	23	-0.990	-7.599	-2.663	1.00	4.83
ATOM	312	HB2	TYR	23	-2.406	-8.540	-2.250	1.00	5.71
ATOM	313	CG	TYR	23	-0.599	-9.294	-1.468	1.00	5.19
ATOM	314	CD1	TYR	23	-1.141	-10.531	-1.148	1.00	5.34
ATOM	315	HD1	TYR	23	-2.212	-10.660	-1.183	1.00	5.46
ATOM	316	CD2	TYR	23	0.776	-9.147	-1.418	1.00	5.53
ATOM	317	HD2	TYR	23	1.205	-8.183	-1.658	1.00	5.77
ATOM	318	CE1	TYR	23	-0.332	-11.592	-0.790	1.00	5.56
ATOM	319	HE1	TYR	23	-0.771	-12.547	-0.545	1.00	5.82
ATOM	320	CE2	TYR	23	1.595	-10.200	-1.059	1.00	5.81
ATOM	321	HE2	TYR	23	2.667	-10.065	-1.025	1.00	6.26
ATOM	322	CZ	TYR	23	1.036	-11.421	-0.748	1.00	5.71
ATOM	323	OH	TYR	23	1.846	-12.475	-0.395	1.00	6.01
ATOM	324	HH	TYR	23	2.160	-12.917	-1.187	1.00	6.05
ATOM	325	C	TYR	23	-0.764	-7.219	0.355	1.00	4.08
ATOM	326	O	TYR	23	-0.942	-7.990	1.300	1.00	4.31
ATOM	327	N	CYS	24	0.305	-6.427	0.252	1.00	3.99
ATOM	328	HN	CYS	24	0.376	-5.836	-0.520	1.00	4.20
ATOM	329	CA	CYS	24	1.379	-6.392	1.250	1.00	3.97

ATOM	330	HA	CYS	24	2.008	-5.546	0.990	1.00	3.54
ATOM	331	HB1	CYS	24	0.849	-7.090	3.203	1.00	4.45
ATOM	332	HB2	CYS	24	-0.159	-5.779	2.608	1.00	4.72
ATOM	333	C	CYS	24	2.230	-7.664	1.271	1.00	4.56
ATOM	334	O	CYS	24	1.702	-8.775	1.300	1.00	4.95
ATOM	335	CB	CYS	24	0.841	-6.167	2.655	1.00	4.21
ATOM	336	SG	CYS	24	1.818	-4.991	3.578	1.00	3.97
ATOM	337	N	ARG	25	3.553	-7.485	1.306	1.00	4.81
ATOM	338	HN	ARG	25	3.910	-6.572	1.315	1.00	4.70
ATOM	339	CA	ARG	25	4.485	-8.614	1.370	1.00	5.41
ATOM	340	HA	ARG	25	4.148	-9.364	0.676	1.00	5.78
ATOM	341	CB	ARG	25	5.903	-8.174	0.986	1.00	5.35
ATOM	342	HB1	ARG	25	6.586	-8.982	1.206	1.00	5.48
ATOM	343	HB2	ARG	25	6.173	-7.317	1.584	1.00	5.55
ATOM	344	CG	ARG	25	6.065	-7.802	-0.478	1.00	5.19
ATOM	345	HG1	ARG	25	5.093	-7.787	-0.945	1.00	5.37
ATOM	346	HG2	ARG	25	6.687	-8.541	-0.961	1.00	5.36
ATOM	347	CD	ARG	25	6.712	-6.433	-0.636	1.00	5.12
ATOM	348	HD1	ARG	25	6.606	-5.890	0.292	1.00	5.22
ATOM	349	HD2	ARG	25	6.201	-5.899	-1.423	1.00	4.97
ATOM	350	NE	ARG	25	8.134	-6.527	-0.973	1.00	5.60
ATOM	351	HE	ARG	25	8.367	-6.596	-1.923	1.00	5.89
ATOM	352	CZ	ARG	25	9.125	-6.525	-0.074	1.00	5.97
ATOM	353	NH1	ARG	25	8.863	-6.428	1.227	1.00	5.99
ATOM	354	HH11	ARG	25	7.920	-6.356	1.546	1.00	5.75
ATOM	355	HH12	ARG	25	9.614	-6.428	1.888	1.00	6.45
ATOM	356	NH2	ARG	25	10.386	-6.622	-0.477	1.00	6.62
ATOM	357	HH21	ARG	25	10.595	-6.697	-1.451	1.00	6.89
ATOM	358	HH22	ARG	25	11.127	-6.620	0.194	1.00	6.99
ATOM	359	C	ARG	25	4.514	-9.217	2.773	1.00	5.75
ATOM	360	O	ARG	25	4.289	-10.415	2.952	1.00	6.36
ATOM	361	N	PHE	26	4.802	-8.369	3.759	1.00	5.44
ATOM	362	HN	PHE	26	4.967	-7.433	3.534	1.00	4.97
ATOM	363	CA	PHE	26	4.879	-8.793	5.158	1.00	5.87
ATOM	364	HA	PHE	26	4.990	-9.862	5.151	1.00	6.37
ATOM	365	CB	PHE	26	6.121	-8.187	5.838	1.00	5.87
ATOM	366	HB1	PHE	26	6.993	-8.742	5.519	1.00	6.14
ATOM	367	HB2	PHE	26	6.026	-8.279	6.907	1.00	5.89
ATOM	368	CG	PHE	26	6.361	-6.740	5.515	1.00	5.68

ATOM	369	CD1	PHE	26	6.033	-5.748	6.420	1.00	5.91
ATOM	370	HD1	PHE	26	5.595	-6.022	7.368	1.00	6.30
ATOM	371	CD2	PHE	26	6.920	-6.381	4.303	1.00	5.59
ATOM	372	HD2	PHE	26	7.172	-7.156	3.593	1.00	5.76
ATOM	373	CE1	PHE	26	6.259	-4.419	6.122	1.00	5.89
ATOM	374	HE1	PHE	26	5.999	-3.653	6.837	1.00	6.28
ATOM	375	CE2	PHE	26	7.150	-5.054	3.995	1.00	5.57
ATOM	376	HE2	PHE	26	7.587	-4.787	3.044	1.00	5.74
ATOM	377	CZ	PHE	26	6.819	-4.071	4.907	1.00	5.65
ATOM	378	HZ	PHE	26	6.997	-3.032	4.670	1.00	5.75
ATOM	379	C	PHE	26	3.576	-8.452	5.906	1.00	5.78
ATOM	380	O	PHE	26	2.491	-8.694	5.373	1.00	5.69
ATOM	381	N	PHE	27	3.669	-7.902	7.131	1.00	5.97
ATOM	382	HN	PHE	27	4.549	-7.733	7.517	1.00	6.18
ATOM	383	CA	PHE	27	2.477	-7.552	7.910	1.00	6.04
ATOM	384	HA	PHE	27	1.921	-8.463	8.076	1.00	6.43
ATOM	385	CB	PHE	27	2.876	-6.962	9.273	1.00	6.49
ATOM	386	HB1	PHE	27	2.391	-6.006	9.402	1.00	6.27
ATOM	387	HB2	PHE	27	3.945	-6.822	9.299	1.00	6.51
ATOM	388	CG	PHE	27	2.497	-7.831	10.440	1.00	7.32
ATOM	389	CD1	PHE	27	3.134	-9.042	10.657	1.00	7.85
ATOM	390	HD1	PHE	27	3.910	-9.363	9.977	1.00	7.76
ATOM	391	CD2	PHE	27	1.503	-7.433	11.321	1.00	7.78
ATOM	392	HD2	PHE	27	1.000	-6.491	11.161	1.00	7.65
ATOM	393	CE1	PHE	27	2.787	-9.840	11.731	1.00	8.65
ATOM	394	HE1	PHE	27	3.290	-10.783	11.889	1.00	9.17
ATOM	395	CE2	PHE	27	1.151	-8.227	12.395	1.00	8.58
ATOM	396	HE2	PHE	27	0.375	-7.905	13.074	1.00	9.03
ATOM	397	CZ	PHE	27	1.794	-9.432	12.601	1.00	8.94
ATOM	398	HZ	PHE	27	1.521	-10.054	13.440	1.00	9.59
ATOM	399	C	PHE	27	1.587	-6.578	7.130	1.00	5.30
ATOM	400	O	PHE	27	0.738	-7.010	6.348	1.00	5.50
ATOM	401	N	ASN	28	1.790	-5.270	7.320	1.00	4.68
ATOM	402	HN	ASN	28	2.489	-4.973	7.933	1.00	4.87
ATOM	403	CA	ASN	28	1.011	-4.272	6.600	1.00	3.97
ATOM	404	HA	ASN	28	0.924	-4.630	5.591	1.00	3.66
ATOM	405	CB	ASN	28	-0.399	-4.127	7.180	1.00	4.05
ATOM	406	HB1	ASN	28	-0.734	-3.109	7.042	1.00	4.27
ATOM	407	HB2	ASN	28	-0.372	-4.353	8.235	1.00	4.30

ATOM	408	CG	ASN	28	-1.411	-5.060	6.513	1.00	4.05
ATOM	409	OD1	ASN	28	-2.267	-5.631	7.187	1.00	4.69
ATOM	410	ND2	ASN	28	-1.320	-5.227	5.185	1.00	3.69
ATOM	411	HD21	ASN	28	-0.620	-4.749	4.698	1.00	3.34
ATOM	412	HD22	ASN	28	-1.961	-5.829	4.749	1.00	4.01
ATOM	413	C	ASN	28	1.730	-2.924	6.548	1.00	3.77
ATOM	414	O	ASN	28	1.560	-2.065	7.416	1.00	3.98
ATOM	415	N	ALA	29	2.500	-2.756	5.480	1.00	3.54
ATOM	416	HN	ALA	29	2.541	-3.464	4.828	1.00	3.55
ATOM	417	CA	ALA	29	3.237	-1.556	5.187	1.00	3.45
ATOM	418	HA	ALA	29	2.593	-0.713	5.396	1.00	3.44
ATOM	419	CB	ALA	29	4.495	-1.408	5.996	1.00	3.85
ATOM	420	HB1	ALA	29	4.945	-0.462	5.737	1.00	4.00
ATOM	421	HB2	ALA	29	5.173	-2.212	5.761	1.00	3.97
ATOM	422	HB3	ALA	29	4.255	-1.422	7.047	1.00	4.27
ATOM	423	C	ALA	29	3.563	-1.564	3.712	1.00	3.33
ATOM	424	O	ALA	29	4.201	-2.484	3.193	1.00	4.12
ATOM	425	N	PHE	30	3.068	-0.553	3.063	1.00	2.67
ATOM	426	HN	PHE	30	2.558	0.083	3.580	1.00	2.45
ATOM	427	CA	PHE	30	3.209	-0.378	1.613	1.00	2.74
ATOM	428	HA	PHE	30	2.894	0.626	1.371	1.00	2.83
ATOM	429	CB	PHE	30	4.669	-0.566	1.171	1.00	3.35
ATOM	430	HB1	PHE	30	4.796	-0.143	0.185	1.00	3.67
ATOM	431	HB2	PHE	30	4.891	-1.622	1.133	1.00	3.59
ATOM	432	CG	PHE	30	5.673	0.083	2.088	1.00	3.86
ATOM	433	CD1	PHE	30	5.441	1.346	2.615	1.00	4.35
ATOM	434	HD1	PHE	30	4.529	1.867	2.361	1.00	4.45
ATOM	435	CD2	PHE	30	6.847	-0.573	2.424	1.00	4.38
ATOM	436	HD2	PHE	30	7.040	-1.557	2.020	1.00	4.49
ATOM	437	CE1	PHE	30	6.360	1.938	3.459	1.00	5.11
ATOM	438	HE1	PHE	30	6.167	2.922	3.862	1.00	5.69
ATOM	439	CE2	PHE	30	7.769	0.016	3.266	1.00	5.15
ATOM	440	HE2	PHE	30	8.681	-0.505	3.520	1.00	5.77
ATOM	441	CZ	PHE	30	7.525	1.273	3.785	1.00	5.42
ATOM	442	HZ	PHE	30	8.245	1.735	4.444	1.00	6.14
ATOM	443	C	PHE	30	2.287	-1.369	0.894	1.00	2.53
ATOM	444	O	PHE	30	2.704	-2.085	-0.011	1.00	3.13
ATOM	445	N	CYS	31	1.034	-1.406	1.362	1.00	2.06
ATOM	446	HN	CYS	31	0.818	-0.818	2.101	1.00	2.12

ATOM	447	CA	CYS	31	-0.017	-2.306	0.854	1.00	1.97
ATOM	448	HA	CYS	31	0.224	-2.580	-0.153	1.00	2.16
ATOM	449	HB1	CYS	31	0.505	-4.333	1.205	1.00	2.78
ATOM	450	HB2	CYS	31	-1.059	-3.866	1.863	1.00	2.85
ATOM	451	C	CYS	31	-1.410	-1.651	0.917	1.00	1.56
ATOM	452	O	CYS	31	-1.944	-1.466	2.011	1.00	2.04
ATOM	453	CB	CYS	31	-0.037	-3.553	1.710	1.00	2.51
ATOM	454	SG	CYS	31	0.725	-3.308	3.341	1.00	2.99
ATOM	455	N	TYR	32	-1.997	-1.284	-0.236	1.00	1.35
ATOM	456	HN	TYR	32	-1.539	-1.446	-1.075	1.00	1.77
ATOM	457	CA	TYR	32	-3.327	-0.635	-0.238	1.00	1.25
ATOM	458	HA	TYR	32	-3.903	-1.138	0.503	1.00	1.48
ATOM	459	CB	TYR	32	-3.228	0.842	0.167	1.00	1.68
ATOM	460	HB1	TYR	32	-4.003	1.065	0.885	1.00	1.99
ATOM	461	HB2	TYR	32	-3.365	1.461	-0.707	1.00	2.16
ATOM	462	CG	TYR	32	-1.909	1.201	0.781	1.00	2.00
ATOM	463	CD1	TYR	32	-0.740	1.028	0.066	1.00	2.67
ATOM	464	HD1	TYR	32	-0.802	0.652	-0.942	1.00	3.10
ATOM	465	CD2	TYR	32	-1.829	1.678	2.069	1.00	2.45
ATOM	466	HD2	TYR	32	-2.743	1.831	2.638	1.00	2.72
ATOM	467	CE1	TYR	32	0.484	1.317	0.605	1.00	3.29
ATOM	468	HE1	TYR	32	1.382	1.166	0.001	1.00	3.99
ATOM	469	CE2	TYR	32	-0.612	1.976	2.629	1.00	3.14
ATOM	470	HE2	TYR	32	-0.576	2.335	3.635	1.00	3.79
ATOM	471	CZ	TYR	32	0.550	1.794	1.900	1.00	3.39
ATOM	472	OH	TYR	32	1.768	2.097	2.465	1.00	4.22
ATOM	473	HH	TYR	32	1.978	3.019	2.301	1.00	4.50
ATOM	474	C	TYR	32	-4.105	-0.726	-1.553	1.00	1.55
ATOM	475	O	TYR	32	-5.142	-1.384	-1.621	1.00	2.20
ATOM	476	N	CYS	33	-3.660	0.019	-2.571	1.00	2.04
ATOM	477	HN	CYS	33	-2.872	0.584	-2.442	1.00	2.31
ATOM	478	CA	CYS	33	-4.394	0.077	-3.831	1.00	2.83
ATOM	479	HA	CYS	33	-5.173	-0.658	-3.776	1.00	3.09
ATOM	480	HB1	CYS	33	-5.053	1.928	-2.929	1.00	3.26
ATOM	481	HB2	CYS	33	-6.070	1.345	-4.241	1.00	3.20
ATOM	482	C	CYS	33	-3.578	-0.177	-5.137	1.00	3.55
ATOM	483	O	CYS	33	-4.167	-0.120	-6.219	1.00	4.43
ATOM	484	CB	CYS	33	-5.055	1.457	-3.915	1.00	3.01
ATOM	485	SG	CYS	33	-4.215	2.580	-5.069	1.00	3.40

ATOM	486	N	ARG	34	-2.255	-0.404	-5.084	1.00	3.34
ATOM	487	HN	ARG	34	-1.777	-0.399	-4.203	1.00	2.80
ATOM	488	CA	ARG	34	-1.467	-0.586	-6.333	1.00	4.11
ATOM	489	HA	ARG	34	-2.019	-0.101	-7.125	1.00	4.69
ATOM	490	CB	ARG	34	-0.137	0.169	-6.148	1.00	4.21
ATOM	491	HB1	ARG	34	0.270	-0.122	-5.193	1.00	4.15
ATOM	492	HB2	ARG	34	-0.360	1.220	-6.109	1.00	4.16
ATOM	493	CG	ARG	34	0.964	-0.004	-7.166	1.00	4.98
ATOM	494	HG1	ARG	34	0.969	0.843	-7.835	1.00	5.57
ATOM	495	HG2	ARG	34	0.809	-0.914	-7.721	1.00	5.02
ATOM	496	CD	ARG	34	2.301	-0.067	-6.425	1.00	5.32
ATOM	497	HD1	ARG	34	2.654	-1.087	-6.429	1.00	5.23
ATOM	498	HD2	ARG	34	2.143	0.253	-5.385	1.00	5.43
ATOM	499	NE	ARG	34	3.314	0.793	-7.034	1.00	6.08
ATOM	500	HE	ARG	34	3.149	1.112	-7.946	1.00	6.18
ATOM	501	CZ	ARG	34	4.441	1.162	-6.420	1.00	6.88
ATOM	502	NH1	ARG	34	4.701	0.747	-5.183	1.00	7.12
ATOM	503	HH11	ARG	34	4.052	0.155	-4.705	1.00	6.71
ATOM	504	HH12	ARG	34	5.546	1.028	-4.728	1.00	7.87
ATOM	505	NH2	ARG	34	5.313	1.944	-7.048	1.00	7.69
ATOM	506	HH21	ARG	34	5.125	2.257	-7.979	1.00	7.76
ATOM	507	HH22	ARG	34	6.157	2.222	-6.589	1.00	8.36
ATOM	508	C	ARG	34	-1.287	-2.074	-6.731	1.00	4.36
ATOM	509	O	ARG	34	-2.259	-2.732	-7.106	1.00	4.73
ATOM	510	N	LYS	35	-0.057	-2.590	-6.668	1.00	4.53
ATOM	511	HN	LYS	35	0.674	-2.028	-6.364	1.00	4.61
ATOM	512	CA	LYS	35	0.255	-3.975	-7.032	1.00	4.93
ATOM	513	HA	LYS	35	-0.509	-4.613	-6.607	1.00	5.03
ATOM	514	CB	LYS	35	0.293	-4.170	-8.558	1.00	5.89
ATOM	515	HB1	LYS	35	-0.714	-4.346	-8.907	1.00	6.19
ATOM	516	HB2	LYS	35	0.895	-5.039	-8.780	1.00	6.25
ATOM	517	CG	LYS	35	0.863	-2.989	-9.331	1.00	6.31
ATOM	518	HG1	LYS	35	0.552	-2.075	-8.852	1.00	6.46
ATOM	519	HG2	LYS	35	0.479	-3.014	-10.339	1.00	6.32
ATOM	520	CD	LYS	35	2.384	-3.025	-9.387	1.00	6.93
ATOM	521	HD1	LYS	35	2.689	-3.525	-10.295	1.00	7.08
ATOM	522	HD2	LYS	35	2.755	-3.570	-8.532	1.00	7.20
ATOM	523	CE	LYS	35	2.972	-1.623	-9.373	1.00	7.41
ATOM	524	HE1	LYS	35	3.647	-1.538	-8.533	1.00	7.65

ATOM	525	HE2	LYS	35	2.169	-0.911	-9.260	1.00	7.56
ATOM	526	NZ	LYS	35	3.716	-1.314	-10.626	1.00	7.75
ATOM	527	HZ1	LYS	35	3.082	-1.396	-11.447	1.00	7.81
ATOM	528	HZ2	LYS	35	4.509	-1.975	-10.746	1.00	8.01
ATOM	529	HZ3	LYS	35	4.091	-0.344	-10.588	1.00	7.95
ATOM	530	C	LYS	35	1.596	-4.335	-6.418	1.00	4.53
ATOM	531	O	LYS	35	2.432	-3.453	-6.214	1.00	4.71
ATOM	532	N	LEU	36	1.805	-5.602	-6.086	1.00	4.30
ATOM	533	HN	LEU	36	1.130	-6.257	-6.243	1.00	4.47
ATOM	534	CA	LEU	36	3.036	-5.989	-5.453	1.00	4.13
ATOM	535	HA	LEU	36	3.354	-5.105	-4.916	1.00	3.77
ATOM	536	CB	LEU	36	2.843	-7.055	-4.416	1.00	4.20
ATOM	537	HB1	LEU	36	3.786	-7.540	-4.232	1.00	4.50
ATOM	538	HB2	LEU	36	2.120	-7.776	-4.770	1.00	4.66
ATOM	539	CG	LEU	36	2.360	-6.422	-3.135	1.00	3.78
ATOM	540	HG	LEU	36	2.622	-5.367	-3.189	1.00	3.57
ATOM	541	CD1	LEU	36	0.849	-6.524	-3.055	1.00	4.07
ATOM	542	HD11	LEU	36	0.535	-6.468	-2.028	1.00	4.35
ATOM	543	HD12	LEU	36	0.528	-7.464	-3.481	1.00	4.26
ATOM	544	HD13	LEU	36	0.405	-5.712	-3.610	1.00	4.30
ATOM	545	CD2	LEU	36	3.040	-7.016	-1.907	1.00	4.09
ATOM	546	HD21	LEU	36	4.020	-6.576	-1.793	1.00	4.26
ATOM	547	HD22	LEU	36	3.138	-8.084	-2.027	1.00	4.48
ATOM	548	HD23	LEU	36	2.447	-6.802	-1.026	1.00	4.28
ATOM	549	C	LEU	36	4.137	-6.293	-6.405	1.00	4.76
ATOM	550	O	LEU	36	4.441	-7.429	-6.775	1.00	5.31
ATOM	551	N	GLY	37	4.752	-5.198	-6.707	1.00	4.97
ATOM	552	HN	GLY	37	4.412	-4.392	-6.286	1.00	4.82
ATOM	553	CA	GLY	37	5.915	-5.144	-7.545	1.00	5.70
ATOM	554	HA1	GLY	37	5.776	-4.401	-8.313	1.00	6.04
ATOM	555	HA2	GLY	37	6.065	-6.105	-7.993	1.00	6.19
ATOM	556	C	GLY	37	7.120	-4.776	-6.704	1.00	5.54
ATOM	557	O	GLY	37	7.963	-3.978	-7.115	1.00	6.08
ATOM	558	N	THR	38	7.161	-5.356	-5.495	1.00	5.00
ATOM	559	HN	THR	38	6.446	-5.959	-5.236	1.00	4.79
ATOM	560	CA	THR	38	8.202	-5.119	-4.526	1.00	4.96
ATOM	561	HA	THR	38	8.007	-5.786	-3.699	1.00	5.16
ATOM	562	CB	THR	38	9.591	-5.445	-5.075	1.00	5.78
ATOM	563	HB	THR	38	10.154	-4.531	-5.184	1.00	6.05

ATOM	564	OG1	THR	38	9.526	-6.095	-6.337	1.00	6.18
ATOM	565	HG1	THR	38	9.079	-6.941	-6.244	1.00	6.46
ATOM	566	CG2	THR	38	10.345	-6.343	-4.136	1.00	6.38
ATOM	567	HG21	THR	38	9.756	-7.232	-3.961	1.00	6.46
ATOM	568	HG22	THR	38	10.500	-5.827	-3.199	1.00	6.55
ATOM	569	HG23	THR	38	11.296	-6.614	-4.570	1.00	6.89
ATOM	570	C	THR	38	8.149	-3.700	-3.987	1.00	4.17
ATOM	571	O	THR	38	8.747	-2.781	-4.553	1.00	4.31
ATOM	572	N	ALA	39	7.460	-3.542	-2.852	1.00	3.68
ATOM	573	HN	ALA	39	7.044	-4.326	-2.437	1.00	3.92
ATOM	574	CA	ALA	39	7.370	-2.242	-2.190	1.00	3.19
ATOM	575	HA	ALA	39	6.953	-1.533	-2.893	1.00	3.39
ATOM	576	CB	ALA	39	6.459	-2.305	-0.952	1.00	2.97
ATOM	577	HB1	ALA	39	5.453	-1.989	-1.214	1.00	3.17
ATOM	578	HB2	ALA	39	6.845	-1.652	-0.184	1.00	3.05
ATOM	579	HB3	ALA	39	6.429	-3.317	-0.578	1.00	3.21
ATOM	580	C	ALA	39	8.789	-1.809	-1.820	1.00	3.22
ATOM	581	O	ALA	39	9.116	-0.625	-1.846	1.00	3.39
ATOM	582	N	MET	40	9.628	-2.825	-1.539	1.00	3.43
ATOM	583	HN	MET	40	9.270	-3.743	-1.595	1.00	3.65
ATOM	584	CA	MET	40	11.056	-2.668	-1.215	1.00	3.68
ATOM	585	HA	MET	40	11.340	-3.533	-0.635	1.00	3.98
ATOM	586	CB	MET	40	11.888	-2.677	-2.505	1.00	4.33
ATOM	587	HB1	MET	40	12.568	-1.837	-2.491	1.00	4.41
ATOM	588	HB2	MET	40	11.222	-2.575	-3.350	1.00	4.46
ATOM	589	CG	MET	40	12.704	-3.945	-2.692	1.00	5.16
ATOM	590	HG1	MET	40	12.841	-4.118	-3.751	1.00	5.45
ATOM	591	HG2	MET	40	12.162	-4.774	-2.262	1.00	5.31
ATOM	592	SD	MET	40	14.325	-3.847	-1.907	1.00	6.00
ATOM	593	CE	MET	40	15.309	-3.173	-3.244	1.00	6.60
ATOM	594	HE1	MET	40	15.124	-3.736	-4.146	1.00	6.92
ATOM	595	HE2	MET	40	16.356	-3.239	-2.987	1.00	6.56
ATOM	596	HE3	MET	40	15.041	-2.139	-3.404	1.00	7.01
ATOM	597	C	MET	40	11.386	-1.423	-0.381	1.00	3.37
ATOM	598	O	MET	40	11.559	-1.513	0.835	1.00	3.68
ATOM	599	N	ASN	41	11.510	-0.272	-1.048	1.00	3.35
ATOM	600	HN	ASN	41	11.386	-0.269	-2.021	1.00	3.59
ATOM	601	CA	ASN	41	11.864	0.977	-0.377	1.00	3.57
ATOM	602	HA	ASN	41	12.586	0.745	0.390	1.00	4.02

ATOM	603	CB	ASN	41	12.503	1.948	-1.377	1.00	4.36
ATOM	604	HB1	ASN	41	12.506	2.941	-0.952	1.00	4.61
ATOM	605	HB2	ASN	41	11.920	1.953	-2.286	1.00	4.37
ATOM	606	CG	ASN	41	13.932	1.569	-1.727	1.00	5.30
ATOM	607	OD1	ASN	41	14.191	0.473	-2.226	1.00	5.84
ATOM	608	ND2	ASN	41	14.868	2.477	-1.470	1.00	5.81
ATOM	609	HD21	ASN	41	14.592	3.331	-1.074	1.00	5.65
ATOM	610	HD22	ASN	41	15.798	2.256	-1.685	1.00	6.50
ATOM	611	C	ASN	41	10.651	1.636	0.277	1.00	3.02
ATOM	612	O	ASN	41	9.507	1.390	-0.108	1.00	3.02
ATOM	613	N	PRO	42	10.898	2.490	1.291	1.00	3.12
ATOM	614	CA	PRO	42	9.833	3.192	2.018	1.00	3.15
ATOM	615	HA	PRO	42	9.154	2.496	2.484	1.00	3.54
ATOM	616	CB	PRO	42	10.588	3.974	3.102	1.00	4.06
ATOM	617	HB1	PRO	42	10.553	3.428	4.033	1.00	4.67
ATOM	618	HB2	PRO	42	10.133	4.945	3.230	1.00	4.13
ATOM	619	CG	PRO	42	11.983	4.089	2.593	1.00	4.46
ATOM	620	HG1	PRO	42	12.673	4.160	3.421	1.00	5.17
ATOM	621	HG2	PRO	42	12.072	4.956	1.954	1.00	4.81
ATOM	622	CD	PRO	42	12.235	2.834	1.810	1.00	3.83
ATOM	623	HD1	PRO	42	12.611	2.055	2.456	1.00	4.33
ATOM	624	HD2	PRO	42	12.927	3.025	1.002	1.00	3.84
ATOM	625	C	PRO	42	9.043	4.145	1.125	1.00	2.55
ATOM	626	O	PRO	42	9.610	5.065	0.531	1.00	2.68
ATOM	627	N	CYS	43	7.731	3.923	1.041	1.00	2.61
ATOM	628	HN	CYS	43	7.340	3.178	1.541	1.00	3.08
ATOM	629	CA	CYS	43	6.862	4.768	0.230	1.00	2.66
ATOM	630	HA	CYS	43	7.382	5.025	-0.623	1.00	3.10
ATOM	631	HB1	CYS	43	4.942	3.935	0.709	1.00	2.52
ATOM	632	HB2	CYS	43	5.840	3.031	-0.503	1.00	2.27
ATOM	633	C	CYS	43	6.535	6.042	0.962	1.00	3.31
ATOM	634	O	CYS	43	6.796	7.152	0.494	1.00	3.75
ATOM	635	CB	CYS	43	5.579	4.021	-0.159	1.00	2.41
ATOM	636	SG	CYS	43	4.616	4.831	-1.478	1.00	3.14
ATOM	637	N	SER	44	5.994	5.829	2.121	1.00	3.89
ATOM	638	HN	SER	44	5.876	4.902	2.362	1.00	3.89
ATOM	639	CA	SER	44	5.605	6.891	3.055	1.00	4.87
ATOM	640	HA	SER	44	4.903	6.474	3.757	1.00	5.17
ATOM	641	CB	SER	44	6.842	7.366	3.832	1.00	5.33

ATOM	642	HB1	SER	44	7.327	6.513	4.284	1.00	5.78
ATOM	643	HB2	SER	44	6.539	8.054	4.606	1.00	5.38
ATOM	644	OG	SER	44	7.771	8.016	2.981	1.00	5.71
ATOM	645	HG	SER	44	8.433	7.384	2.689	1.00	5.91
ATOM	646	C	SER	44	4.916	8.060	2.338	1.00	5.60
ATOM	647	O	SER	44	4.324	7.887	1.270	1.00	5.82
ATOM	648	N	ARG	45	4.992	9.244	2.946	1.00	6.31
ATOM	649	HN	ARG	45	5.466	9.302	3.798	1.00	6.38
ATOM	650	CA	ARG	45	4.385	10.456	2.392	1.00	7.28
ATOM	651	HA	ARG	45	4.713	11.288	2.997	1.00	7.53
ATOM	652	CB	ARG	45	4.845	10.690	0.944	1.00	7.96
ATOM	653	HB1	ARG	45	4.706	11.733	0.701	1.00	8.31
ATOM	654	HB2	ARG	45	4.231	10.094	0.285	1.00	7.94
ATOM	655	CG	ARG	45	6.301	10.331	0.684	1.00	8.60
ATOM	656	HG1	ARG	45	6.471	10.321	-0.383	1.00	8.78
ATOM	657	HG2	ARG	45	6.498	9.351	1.089	1.00	8.86
ATOM	658	CD	ARG	45	7.254	11.328	1.326	1.00	9.04
ATOM	659	HD1	ARG	45	6.712	11.904	2.062	1.00	9.20
ATOM	660	HD2	ARG	45	7.632	11.990	0.560	1.00	8.91
ATOM	661	NE	ARG	45	8.384	10.664	1.981	1.00	9.74
ATOM	662	HE	ARG	45	8.404	10.675	2.960	1.00	9.93
ATOM	663	CZ	ARG	45	9.375	10.047	1.328	1.00	10.31
ATOM	664	NH1	ARG	45	9.388	10.005	-0.002	1.00	10.31
ATOM	665	HH11	ARG	45	8.653	10.435	-0.525	1.00	9.86
ATOM	666	HH12	ARG	45	10.134	9.541	-0.479	1.00	10.88
ATOM	667	NH2	ARG	45	10.357	9.466	2.011	1.00	11.06
ATOM	668	HH21	ARG	45	10.355	9.490	3.011	1.00	11.21
ATOM	669	HH22	ARG	45	11.100	9.004	1.525	1.00	11.58
ATOM	670	C	ARG	45	2.858	10.387	2.457	1.00	7.69
ATOM	671	O	ARG	45	2.168	10.651	1.468	1.00	8.34
ATOM	672	N	THR	46	2.336	10.036	3.633	1.00	7.60
ATOM	673	HN	THR	46	2.935	9.842	4.379	1.00	7.26
ATOM	674	CA	THR	46	0.895	9.934	3.836	1.00	8.32
ATOM	675	HA	THR	46	0.488	9.396	2.997	1.00	8.41
ATOM	676	CB	THR	46	0.582	9.157	5.122	1.00	8.61
ATOM	677	HB	THR	46	-0.480	9.219	5.314	1.00	8.70
ATOM	678	OG1	THR	46	1.266	9.717	6.233	1.00	8.93
ATOM	679	HG1	THR	46	2.175	9.410	6.243	1.00	9.05
ATOM	680	CG2	THR	46	0.951	7.688	5.037	1.00	8.89

ATOM	681	HG21	THR	46	0.433	7.232	4.206	1.00	9.07
ATOM	682	HG22	THR	46	0.668	7.191	5.954	1.00	9.06
ATOM	683	HG23	THR	46	2.017	7.593	4.891	1.00	9.01
ATOM	684	C	THR	46	0.249	11.319	3.889	1.00	9.05
ATOM	685	OT1	THR	46	0.759	12.187	4.632	1.00	9.34
ATOM	686	OT2	THR	46	-0.761	11.525	3.184	1.00	9.53

Table 5. MARP atomic coordinates in 3 dimensional space determine by NMR at 800 mHz.

ATOM	1	N	CYS	A	1	-12.726	-3.631	3.103	1.00	0.00	N
ATOM	2	CA	CYS	A	1	-11.311	-3.175	3.172	1.00	0.00	C
ATOM	3	C	CYS	A	1	-10.813	-2.734	1.797	1.00	0.00	C
ATOM	4	O	CYS	A	1	-11.608	-2.465	0.895	1.00	0.00	O
ATOM	5	CB	CYS	A	1	-10.421	-4.311	3.710	1.00	0.00	C
ATOM	6	SG	CYS	A	1	-11.306	-5.794	4.301	1.00	0.00	S
ATOM	7	H	CYS	A	1	-13.283	-2.870	2.668	1.00	0.00	H
ATOM	8	HA	CYS	A	1	-11.258	-2.333	3.848	1.00	0.00	H
ATOM	9	1HB	CYS	A	1	-9.754	-4.626	2.925	1.00	0.00	H
ATOM	10	2HB	CYS	A	1	-9.837	-3.932	4.534	1.00	0.00	H
ATOM	11	N	VAL	A	2	-9.494	-2.666	1.644	1.00	0.00	N
ATOM	12	CA	VAL	A	2	-8.887	-2.267	0.382	1.00	0.00	C
ATOM	13	C	VAL	A	2	-8.548	-3.483	-0.461	1.00	0.00	C
ATOM	14	O	VAL	A	2	-8.532	-4.609	0.036	1.00	0.00	O
ATOM	15	CB	VAL	A	2	-7.610	-1.429	0.600	1.00	0.00	C
ATOM	16	CG1	VAL	A	2	-7.264	-0.642	-0.655	1.00	0.00	C
ATOM	17	CG2	VAL	A	2	-7.772	-0.495	1.792	1.00	0.00	C
ATOM	18	H	VAL	A	2	-8.913	-2.896	2.399	1.00	0.00	H
ATOM	19	HA	VAL	A	2	-9.602	-1.668	-0.161	1.00	0.00	H
ATOM	20	HB	VAL	A	2	-6.794	-2.106	0.807	1.00	0.00	H
ATOM	21	1HG1	VAL	A	2	-6.616	0.182	-0.397	1.00	0.00	H
ATOM	22	2HG1	VAL	A	2	-8.171	-0.259	-1.100	1.00	0.00	H
ATOM	23	3HG1	VAL	A	2	-6.763	-1.289	-1.359	1.00	0.00	H
ATOM	24	1HG2	VAL	A	2	-8.466	0.293	1.540	1.00	0.00	H
ATOM	25	2HG2	VAL	A	2	-6.814	-0.065	2.043	1.00	0.00	H
ATOM	26	3HG2	VAL	A	2	-8.150	-1.052	2.637	1.00	0.00	H
ATOM	27	N	ARG	A	3	-8.292	-3.256	-1.740	1.00	0.00	N
ATOM	28	CA	ARG	A	3	-7.971	-4.344	-2.645	1.00	0.00	C

ATOM	29	C	ARG	A	3	-6.520	-4.785	-2.479	1.00	0.00	C
ATOM	30	O	ARG	A	3	-5.866	-4.443	-1.495	1.00	0.00	O
ATOM	31	CB	ARG	A	3	-8.238	-3.931	-4.093	1.00	0.00	C
ATOM	32	CG	ARG	A	3	-9.563	-3.209	-4.286	1.00	0.00	C
ATOM	33	CD	ARG	A	3	-10.155	-3.490	-5.657	1.00	0.00	C
ATOM	34	NE	ARG	A	3	-11.422	-2.790	-5.860	1.00	0.00	N
ATOM	35	CZ	ARG	A	3	-11.970	-2.581	-7.054	1.00	0.00	C
ATOM	36	NH1	ARG	A	3	-11.368	-3.017	-8.154	1.00	0.00	N
ATOM	37	NH2	ARG	A	3	-13.123	-1.934	-7.151	1.00	0.00	N
ATOM	38	H	ARG	A	3	-8.331	-2.339	-2.086	1.00	0.00	H
ATOM	39	HA	ARG	A	3	-8.617	-5.168	-2.389	1.00	0.00	H
ATOM	40	1HB	ARG	A	3	-7.445	-3.274	-4.421	1.00	0.00	H
ATOM	41	2HB	ARG	A	3	-8.241	-4.815	-4.714	1.00	0.00	H
ATOM	42	1HG	ARG	A	3	-10.256	-3.545	-3.529	1.00	0.00	H
ATOM	43	2HG	ARG	A	3	-9.401	-2.148	-4.183	1.00	0.00	H
ATOM	44	1HD	ARG	A	3	-9.454	-3.168	-6.411	1.00	0.00	H
ATOM	45	2HD	ARG	A	3	-10.323	-4.553	-5.752	1.00	0.00	H
ATOM	46	HE	ARG	A	3	-11.888	-2.458	-5.064	1.00	0.00	H
ATOM	47	1HH1	ARG	A	3	-10.497	-3.506	-8.088	1.00	0.00	H
ATOM	48	2HH1	ARG	A	3	-11.784	-2.858	-9.049	1.00	0.00	H
ATOM	49	1HH2	ARG	A	3	-13.582	-1.605	-6.326	1.00	0.00	H
ATOM	50	2HH2	ARG	A	3	-13.534	-1.777	-8.049	1.00	0.00	H
ATOM	51	N	LEU	A	4	-6.023	-5.552	-3.445	1.00	0.00	N
ATOM	52	CA	LEU	A	4	-4.657	-6.049	-3.403	1.00	0.00	C
ATOM	53	C	LEU	A	4	-3.655	-4.912	-3.579	1.00	0.00	C
ATOM	54	O	LEU	A	4	-2.627	-4.869	-2.904	1.00	0.00	O
ATOM	55	CB	LEU	A	4	-4.453	-7.095	-4.498	1.00	0.00	C
ATOM	56	CG	LEU	A	4	-3.030	-7.636	-4.611	1.00	0.00	C
ATOM	57	CD1	LEU	A	4	-2.800	-8.754	-3.607	1.00	0.00	C
ATOM	58	CD2	LEU	A	4	-2.756	-8.121	-6.027	1.00	0.00	C
ATOM	59	H	LEU	A	4	-6.593	-5.796	-4.201	1.00	0.00	H
ATOM	60	HA	LEU	A	4	-4.498	-6.509	-2.441	1.00	0.00	H
ATOM	61	1HB	LEU	A	4	-5.119	-7.921	-4.303	1.00	0.00	H
ATOM	62	2HB	LEU	A	4	-4.722	-6.653	-5.444	1.00	0.00	H
ATOM	63	HG	LEU	A	4	-2.339	-6.840	-4.390	1.00	0.00	H
ATOM	64	1HD1	LEU	A	4	-1.768	-9.067	-3.646	1.00	0.00	H
ATOM	65	2HD1	LEU	A	4	-3.439	-9.590	-3.847	1.00	0.00	H
ATOM	66	3HD1	LEU	A	4	-3.031	-8.398	-2.613	1.00	0.00	H

ATOM	67	1HD2	LEU	A	4	-3.092	-9.142	-6.129	1.00	0.00	H
ATOM	68	2HD2	LEU	A	4	-1.694	-8.068	-6.226	1.00	0.00	H
ATOM	69	3HD2	LEU	A	4	-3.284	-7.495	-6.731	1.00	0.00	H
ATOM	70	N	HIS	A	5	-3.963	-3.995	-4.487	1.00	0.00	N
ATOM	71	CA	HIS	A	5	-3.096	-2.863	-4.747	1.00	0.00	C
ATOM	72	C	HIS	A	5	-3.884	-1.696	-5.334	1.00	0.00	C
ATOM	73	O	HIS	A	5	-3.679	-1.308	-6.484	1.00	0.00	O
ATOM	74	CB	HIS	A	5	-1.958	-3.263	-5.689	1.00	0.00	C
ATOM	75	CG	HIS	A	5	-2.420	-4.027	-6.891	1.00	0.00	C
ATOM	76	ND1	HIS	A	5	-1.567	-4.754	-7.694	1.00	0.00	N
ATOM	77	CD2	HIS	A	5	-3.655	-4.175	-7.426	1.00	0.00	C
ATOM	78	CE1	HIS	A	5	-2.255	-5.317	-8.670	1.00	0.00	C
ATOM	79	NE2	HIS	A	5	-3.524	-4.982	-8.530	1.00	0.00	N
ATOM	80	H	HIS	A	5	-4.790	-4.078	-4.985	1.00	0.00	H
ATOM	81	HA	HIS	A	5	-2.685	-2.559	-3.808	1.00	0.00	H
ATOM	82	1HB	HIS	A	5	-1.457	-2.372	-6.034	1.00	0.00	H
ATOM	83	2HB	HIS	A	5	-1.256	-3.881	-5.149	1.00	0.00	H
ATOM	84	HD1	HIS	A	5	-0.598	-4.844	-7.567	1.00	0.00	H
ATOM	85	HD2	HIS	A	5	-4.572	-3.740	-7.054	1.00	0.00	H
ATOM	86	HE1	HIS	A	5	-1.850	-5.946	-9.449	1.00	0.00	H
ATOM	87	HE2	HIS	A	5	-4.265	-5.332	-9.068	1.00	0.00	H
ATOM	88	N	GLU	A	6	-4.784	-1.141	-4.532	1.00	0.00	N
ATOM	89	CA	GLU	A	6	-5.605	-0.015	-4.963	1.00	0.00	C
ATOM	90	C	GLU	A	6	-5.739	1.011	-3.845	1.00	0.00	C
ATOM	91	O	GLU	A	6	-5.364	0.752	-2.701	1.00	0.00	O
ATOM	92	CB	GLU	A	6	-6.988	-0.503	-5.397	1.00	0.00	C
ATOM	93	CG	GLU	A	6	-7.710	0.464	-6.322	1.00	0.00	C
ATOM	94	CD	GLU	A	6	-8.816	-0.205	-7.114	1.00	0.00	C
ATOM	95	OE1	GLU	A	6	-8.635	-1.372	-7.517	1.00	0.00	O
ATOM	96	OE2	GLU	A	6	-9.863	0.441	-7.332	1.00	0.00	O
ATOM	97	H	GLU	A	6	-4.899	-1.494	-3.625	1.00	0.00	H
ATOM	98	HA	GLU	A	6	-5.113	0.449	-5.804	1.00	0.00	H
ATOM	99	1HB	GLU	A	6	-6.879	-1.445	-5.911	1.00	0.00	H
ATOM	100	2HB	GLU	A	6	-7.597	-0.651	-4.518	1.00	0.00	H
ATOM	101	1HG	GLU	A	6	-8.142	1.256	-5.727	1.00	0.00	H
ATOM	102	2HG	GLU	A	6	-6.993	0.884	-7.013	1.00	0.00	H
ATOM	103	N	SER	A	7	-6.278	2.179	-4.180	1.00	0.00	N
ATOM	104	CA	SER	A	7	-6.462	3.245	-3.202	1.00	0.00	C

ATOM	105	C	SER	A	7	-7.396	2.797	-2.083	1.00	0.00	C
ATOM	106	O	SER	A	7	-8.345	2.047	-2.316	1.00	0.00	O
ATOM	107	CB	SER	A	7	-7.018	4.497	-3.880	1.00	0.00	C
ATOM	108	OG	SER	A	7	-6.070	5.060	-4.769	1.00	0.00	O
ATOM	109	H	SER	A	7	-6.559	2.327	-5.108	1.00	0.00	H
ATOM	110	HA	SER	A	7	-5.496	3.473	-2.778	1.00	0.00	H
ATOM	111	1HB	SER	A	7	-7.905	4.237	-4.439	1.00	0.00	H
ATOM	112	2HB	SER	A	7	-7.269	5.230	-3.128	1.00	0.00	H
ATOM	113	HG	SER	A	7	-6.144	4.637	-5.629	1.00	0.00	H
ATOM	114	N	CYS	A	8	-7.124	3.260	-0.867	1.00	0.00	N
ATOM	115	CA	CYS	A	8	-7.941	2.904	0.286	1.00	0.00	C
ATOM	116	C	CYS	A	8	-8.771	4.094	0.762	1.00	0.00	C
ATOM	117	O	CYS	A	8	-9.807	3.916	1.401	1.00	0.00	O
ATOM	118	CB	CYS	A	8	-7.058	2.390	1.427	1.00	0.00	C
ATOM	119	SG	CYS	A	8	-5.978	3.656	2.170	1.00	0.00	S
ATOM	120	H	CYS	A	8	-6.353	3.853	-0.744	1.00	0.00	H
ATOM	121	HA	CYS	A	8	-8.611	2.117	-0.017	1.00	0.00	H
ATOM	122	1HB	CYS	A	8	-7.688	1.998	2.211	1.00	0.00	H
ATOM	123	2HB	CYS	A	8	-6.426	1.599	1.051	1.00	0.00	H
ATOM	124	N	LEU	A	9	-8.302	5.303	0.439	1.00	0.00	N
ATOM	125	CA	LEU	A	9	-8.981	6.552	0.818	1.00	0.00	C
ATOM	126	C	LEU	A	9	-9.886	6.374	2.037	1.00	0.00	C
ATOM	127	O	LEU	A	9	-11.044	5.977	1.911	1.00	0.00	O
ATOM	128	CB	LEU	A	9	-9.802	7.079	-0.360	1.00	0.00	C
ATOM	129	CG	LEU	A	9	-10.634	6.025	-1.093	1.00	0.00	C
ATOM	130	CD1	LEU	A	9	-11.893	6.651	-1.675	1.00	0.00	C
ATOM	131	CD2	LEU	A	9	-9.811	5.362	-2.186	1.00	0.00	C
ATOM	132	H	LEU	A	9	-7.471	5.361	-0.072	1.00	0.00	H
ATOM	133	HA	LEU	A	9	-8.219	7.277	1.059	1.00	0.00	H
ATOM	134	1HB	LEU	A	9	-10.469	7.845	0.009	1.00	0.00	H
ATOM	135	2HB	LEU	A	9	-9.124	7.528	-1.071	1.00	0.00	H
ATOM	136	HG	LEU	A	9	-10.935	5.263	-0.391	1.00	0.00	H
ATOM	137	1HD1	LEU	A	9	-11.708	7.693	-1.894	1.00	0.00	H
ATOM	138	2HD1	LEU	A	9	-12.698	6.571	-0.961	1.00	0.00	H
ATOM	139	3HD1	LEU	A	9	-12.164	6.134	-2.583	1.00	0.00	H
ATOM	140	1HD2	LEU	A	9	-8.792	5.249	-1.850	1.00	0.00	H
ATOM	141	2HD2	LEU	A	9	-9.830	5.978	-3.074	1.00	0.00	H
ATOM	142	3HD2	LEU	A	9	-10.227	4.393	-2.413	1.00	0.00	H

ATOM	143	N	GLY	A	10	-9.348	6.673	3.215	1.00	0.00	N
ATOM	144	CA	GLY	A	10	-10.121	6.541	4.436	1.00	0.00	C
ATOM	145	C	GLY	A	10	-9.254	6.529	5.682	1.00	0.00	C
ATOM	146	O	GLY	A	10	-9.723	6.856	6.773	1.00	0.00	O
ATOM	147	H	GLY	A	10	-8.422	6.989	3.254	1.00	0.00	H
ATOM	148	1HA	GLY	A	10	-10.812	7.369	4.502	1.00	0.00	H
ATOM	149	2HA	GLY	A	10	-10.685	5.622	4.393	1.00	0.00	H
ATOM	150	N	GLN	A	11	-7.987	6.152	5.523	1.00	0.00	N
ATOM	151	CA	GLN	A	11	-7.049	6.098	6.646	1.00	0.00	C
ATOM	152	C	GLN	A	11	-7.329	4.901	7.553	1.00	0.00	C
ATOM	153	O	GLN	A	11	-6.435	4.097	7.819	1.00	0.00	O
ATOM	154	CB	GLN	A	11	-7.109	7.391	7.462	1.00	0.00	C
ATOM	155	CG	GLN	A	11	-7.285	8.643	6.616	1.00	0.00	C
ATOM	156	CD	GLN	A	11	-6.672	9.870	7.259	1.00	0.00	C
ATOM	157	OE1	GLN	A	11	-5.574	9.813	7.813	1.00	0.00	O
ATOM	158	NE2	GLN	A	11	-7.382	10.990	7.193	1.00	0.00	N
ATOM	159	H	GLN	A	11	-7.672	5.903	4.630	1.00	0.00	H
ATOM	160	HA	GLN	A	11	-6.059	5.995	6.238	1.00	0.00	H
ATOM	161	1HB	GLN	A	11	-7.936	7.326	8.146	1.00	0.00	H
ATOM	162	2HB	GLN	A	11	-6.193	7.488	8.025	1.00	0.00	H
ATOM	163	1HG	GLN	A	11	-6.813	8.483	5.658	1.00	0.00	H
ATOM	164	2HG	GLN	A	11	-8.341	8.817	6.471	1.00	0.00	H
ATOM	165	1HE2	GLN	A	11	-8.249	10.961	6.738	1.00	0.00	H
ATOM	166	2HE2	GLN	A	11	-7.010	11.799	7.601	1.00	0.00	H
ATOM	167	N	GLN	A	12	-8.564	4.785	8.029	1.00	0.00	N
ATOM	168	CA	GLN	A	12	-8.941	3.684	8.907	1.00	0.00	C
ATOM	169	C	GLN	A	12	-9.394	2.459	8.111	1.00	0.00	C
ATOM	170	O	GLN	A	12	-9.943	1.514	8.677	1.00	0.00	O
ATOM	171	CB	GLN	A	12	-10.056	4.121	9.865	1.00	0.00	C
ATOM	172	CG	GLN	A	12	-11.051	5.103	9.266	1.00	0.00	C
ATOM	173	CD	GLN	A	12	-11.598	4.640	7.930	1.00	0.00	C
ATOM	174	OE1	GLN	A	12	-10.903	4.677	6.915	1.00	0.00	O
ATOM	175	NE2	GLN	A	12	-12.851	4.199	7.924	1.00	0.00	N
ATOM	176	H	GLN	A	12	-9.236	5.452	7.790	1.00	0.00	H
ATOM	177	HA	GLN	A	12	-8.072	3.415	9.485	1.00	0.00	H
ATOM	178	1HB	GLN	A	12	-10.599	3.248	10.177	1.00	0.00	H
ATOM	179	2HB	GLN	A	12	-9.606	4.582	10.733	1.00	0.00	H
ATOM	180	1HG	GLN	A	12	-11.877	5.223	9.952	1.00	0.00	H

ATOM	181	2HG	GLN	A	12	-10.559	6.055	9.126	1.00	0.00	H
ATOM	182	1HE2	GLN	A	12	-13.347	4.199	8.771	1.00	0.00	H
ATOM	183	2HE2	GLN	A	12	-13.231	3.892	7.074	1.00	0.00	H
ATOM	184	N	VAL	A	13	-9.160	2.473	6.800	1.00	0.00	N
ATOM	185	CA	VAL	A	13	-9.542	1.354	5.946	1.00	0.00	C
ATOM	186	C	VAL	A	13	-8.322	0.528	5.546	1.00	0.00	C
ATOM	187	O	VAL	A	13	-7.729	0.753	4.492	1.00	0.00	O
ATOM	188	CB	VAL	A	13	-10.263	1.836	4.669	1.00	0.00	C
ATOM	189	CG1	VAL	A	13	-10.752	0.652	3.847	1.00	0.00	C
ATOM	190	CG2	VAL	A	13	-11.419	2.758	5.027	1.00	0.00	C
ATOM	191	H	VAL	A	13	-8.718	3.248	6.399	1.00	0.00	H
ATOM	192	HA	VAL	A	13	-10.221	0.725	6.502	1.00	0.00	H
ATOM	193	HB	VAL	A	13	-9.557	2.393	4.070	1.00	0.00	H
ATOM	194	1HG1	VAL	A	13	-11.816	0.529	3.990	1.00	0.00	H
ATOM	195	2HG1	VAL	A	13	-10.241	-0.244	4.164	1.00	0.00	H
ATOM	196	3HG1	VAL	A	13	-10.548	0.831	2.801	1.00	0.00	H
ATOM	197	1HG2	VAL	A	13	-11.116	3.785	4.886	1.00	0.00	H
ATOM	198	2HG2	VAL	A	13	-11.696	2.604	6.059	1.00	0.00	H
ATOM	199	3HG2	VAL	A	13	-12.264	2.543	4.391	1.00	0.00	H
ATOM	200	N	PRO	A	14	-7.930	-0.446	6.386	1.00	0.00	N
ATOM	201	CA	PRO	A	14	-6.777	-1.305	6.110	1.00	0.00	C
ATOM	202	C	PRO	A	14	-7.034	-2.258	4.950	1.00	0.00	C
ATOM	203	O	PRO	A	14	-8.181	-2.502	4.578	1.00	0.00	O
ATOM	204	CB	PRO	A	14	-6.591	-2.086	7.411	1.00	0.00	C
ATOM	205	CG	PRO	A	14	-7.935	-2.085	8.053	1.00	0.00	C
ATOM	206	CD	PRO	A	14	-8.582	-0.784	7.665	1.00	0.00	C
ATOM	207	HA	PRO	A	14	-5.889	-0.723	5.908	1.00	0.00	H
ATOM	208	1HB	PRO	A	14	-6.261	-3.090	7.187	1.00	0.00	H
ATOM	209	2HB	PRO	A	14	-5.859	-1.592	8.030	1.00	0.00	H
ATOM	210	1HG	PRO	A	14	-8.518	-2.917	7.685	1.00	0.00	H
ATOM	211	2HG	PRO	A	14	-7.831	-2.146	9.126	1.00	0.00	H
ATOM	212	1HD	PRO	A	14	-9.646	-0.916	7.532	1.00	0.00	H
ATOM	213	2HD	PRO	A	14	-8.384	-0.027	8.409	1.00	0.00	H
ATOM	214	N	CYS	A	15	-5.961	-2.791	4.378	1.00	0.00	N
ATOM	215	CA	CYS	A	15	-6.075	-3.717	3.259	1.00	0.00	C
ATOM	216	C	CYS	A	15	-6.799	-4.995	3.672	1.00	0.00	C
ATOM	217	O	CYS	A	15	-6.585	-5.518	4.766	1.00	0.00	O
ATOM	218	CB	CYS	A	15	-4.694	-4.058	2.701	1.00	0.00	C

ATOM	219	SG	CYS	A	15	-4.736	-4.924	1.101	1.00	0.00	S
ATOM	220	H	CYS	A	15	-5.072	-2.557	4.720	1.00	0.00	H
ATOM	221	HA	CYS	A	15	-6.652	-3.229	2.487	1.00	0.00	H
ATOM	222	1HB	CYS	A	15	-4.133	-3.147	2.567	1.00	0.00	H
ATOM	223	2HB	CYS	A	15	-4.177	-4.692	3.407	1.00	0.00	H
ATOM	224	N	CYS	A	16	-7.654	-5.495	2.785	1.00	0.00	N
ATOM	225	CA	CYS	A	16	-8.412	-6.714	3.043	1.00	0.00	C
ATOM	226	C	CYS	A	16	-7.515	-7.955	2.994	1.00	0.00	C
ATOM	227	O	CYS	A	16	-7.967	-9.063	3.287	1.00	0.00	O
ATOM	228	CB	CYS	A	16	-9.544	-6.852	2.023	1.00	0.00	C
ATOM	229	SG	CYS	A	16	-11.200	-7.104	2.750	1.00	0.00	S
ATOM	230	H	CYS	A	16	-7.774	-5.030	1.929	1.00	0.00	H
ATOM	231	HA	CYS	A	16	-8.838	-6.639	4.025	1.00	0.00	H
ATOM	232	1HB	CYS	A	16	-9.585	-5.961	1.417	1.00	0.00	H
ATOM	233	2HB	CYS	A	16	-9.334	-7.698	1.391	1.00	0.00	H
ATOM	234	N	ASP	A	17	-6.252	-7.772	2.614	1.00	0.00	N
ATOM	235	CA	ASP	A	17	-5.316	-8.888	2.521	1.00	0.00	C
ATOM	236	C	ASP	A	17	-4.478	-9.026	3.793	1.00	0.00	C
ATOM	237	O	ASP	A	17	-4.012	-8.032	4.356	1.00	0.00	O
ATOM	238	CB	ASP	A	17	-4.398	-8.705	1.311	1.00	0.00	C
ATOM	239	CG	ASP	A	17	-5.169	-8.577	0.014	1.00	0.00	C
ATOM	240	OD1	ASP	A	17	-6.127	-7.776	-0.030	1.00	0.00	O
ATOM	241	OD2	ASP	A	17	-4.818	-9.278	-0.959	1.00	0.00	O
ATOM	242	H	ASP	A	17	-5.944	-6.872	2.383	1.00	0.00	H
ATOM	243	HA	ASP	A	17	-5.895	-9.789	2.384	1.00	0.00	H
ATOM	244	1HB	ASP	A	17	-3.807	-7.811	1.448	1.00	0.00	H
ATOM	245	2HB	ASP	A	17	-3.739	-9.559	1.236	1.00	0.00	H
ATOM	246	N	PRO	A	18	-4.267	-10.271	4.261	1.00	0.00	N
ATOM	247	CA	PRO	A	18	-3.475	-10.536	5.463	1.00	0.00	C
ATOM	248	C	PRO	A	18	-2.086	-9.917	5.375	1.00	0.00	C
ATOM	249	O	PRO	A	18	-1.417	-10.011	4.345	1.00	0.00	O
ATOM	250	CB	PRO	A	18	-3.381	-12.068	5.519	1.00	0.00	C
ATOM	251	CG	PRO	A	18	-3.806	-12.542	4.170	1.00	0.00	C
ATOM	252	CD	PRO	A	18	-4.767	-11.513	3.654	1.00	0.00	C
ATOM	253	HA	PRO	A	18	-3.973	-10.170	6.350	1.00	0.00	H
ATOM	254	1HB	PRO	A	18	-2.363	-12.360	5.737	1.00	0.00	H
ATOM	255	2HB	PRO	A	18	-4.038	-12.441	6.291	1.00	0.00	H
ATOM	256	1HG	PRO	A	18	-2.947	-12.613	3.519	1.00	0.00	H

ATOM	257	2HG	PRO	A	18	-4.293	-13.501	4.254	1.00	0.00	H
ATOM	258	1HD	PRO	A	18	-4.726	-11.462	2.575	1.00	0.00	H
ATOM	259	2HD	PRO	A	18	-5.770	-11.730	3.988	1.00	0.00	H
ATOM	260	N	CYS	A	19	-1.661	-9.276	6.456	1.00	0.00	N
ATOM	261	CA	CYS	A	19	-0.355	-8.631	6.501	1.00	0.00	C
ATOM	262	C	CYS	A	19	-0.270	-7.482	5.495	1.00	0.00	C
ATOM	263	O	CYS	A	19	0.816	-6.977	5.211	1.00	0.00	O
ATOM	264	CB	CYS	A	19	0.753	-9.652	6.222	1.00	0.00	C
ATOM	265	SG	CYS	A	19	2.439	-9.011	6.483	1.00	0.00	S
ATOM	266	H	CYS	A	19	-2.242	-9.230	7.243	1.00	0.00	H
ATOM	267	HA	CYS	A	19	-0.218	-8.232	7.495	1.00	0.00	H
ATOM	268	1HB	CYS	A	19	0.620	-10.502	6.875	1.00	0.00	H
ATOM	269	2HB	CYS	A	19	0.679	-9.980	5.195	1.00	0.00	H
ATOM	270	N	ALA	A	20	-1.418	-7.065	4.959	1.00	0.00	N
ATOM	271	CA	ALA	A	20	-1.450	-5.971	3.995	1.00	0.00	C
ATOM	272	C	ALA	A	20	-2.007	-4.701	4.631	1.00	0.00	C
ATOM	273	O	ALA	A	20	-3.025	-4.738	5.322	1.00	0.00	O
ATOM	274	CB	ALA	A	20	-2.271	-6.363	2.777	1.00	0.00	C
ATOM	275	H	ALA	A	20	-2.259	-7.496	5.218	1.00	0.00	H
ATOM	276	HA	ALA	A	20	-0.435	-5.784	3.671	1.00	0.00	H
ATOM	277	1HB	ALA	A	20	-3.318	-6.393	3.041	1.00	0.00	H
ATOM	278	2HB	ALA	A	20	-1.960	-7.339	2.431	1.00	0.00	H
ATOM	279	3HB	ALA	A	20	-2.119	-5.638	1.992	1.00	0.00	H
ATOM	280	N	THR	A	21	-1.332	-3.580	4.395	1.00	0.00	N
ATOM	281	CA	THR	A	21	-1.761	-2.300	4.948	1.00	0.00	C
ATOM	282	C	THR	A	21	-1.668	-1.197	3.900	1.00	0.00	C
ATOM	283	O	THR	A	21	-0.790	-1.223	3.038	1.00	0.00	O
ATOM	284	CB	THR	A	21	-0.909	-1.938	6.166	1.00	0.00	C
ATOM	285	OG1	THR	A	21	-1.401	-0.766	6.792	1.00	0.00	O
ATOM	286	CG2	THR	A	21	0.547	-1.702	5.830	1.00	0.00	C
ATOM	287	H	THR	A	21	-0.526	-3.615	3.837	1.00	0.00	H
ATOM	288	HA	THR	A	21	-2.791	-2.400	5.257	1.00	0.00	H
ATOM	289	HB	THR	A	21	-0.957	-2.749	6.879	1.00	0.00	H
ATOM	290	HG1	THR	A	21	-1.514	-0.074	6.135	1.00	0.00	H
ATOM	291	1HG2	THR	A	21	0.824	-2.313	4.984	1.00	0.00	H
ATOM	292	2HG2	THR	A	21	1.160	-1.965	6.680	1.00	0.00	H
ATOM	293	3HG2	THR	A	21	0.697	-0.660	5.588	1.00	0.00	H
ATOM	294	N	CYS	A	22	-2.578	-0.231	3.975	1.00	0.00	N

ATOM	295	CA	CYS	A	22	-2.593	0.876	3.026	1.00	0.00	C
ATOM	296	C	CYS	A	22	-1.412	1.813	3.253	1.00	0.00	C
ATOM	297	O	CYS	A	22	-1.294	2.439	4.306	1.00	0.00	O
ATOM	298	CB	CYS	A	22	-3.901	1.658	3.132	1.00	0.00	C
ATOM	299	SG	CYS	A	22	-4.104	2.941	1.855	1.00	0.00	S
ATOM	300	H	CYS	A	22	-3.254	-0.263	4.684	1.00	0.00	H
ATOM	301	HA	CYS	A	22	-2.519	0.461	2.035	1.00	0.00	H
ATOM	302	1HB	CYS	A	22	-4.731	0.972	3.045	1.00	0.00	H
ATOM	303	2HB	CYS	A	22	-3.940	2.141	4.095	1.00	0.00	H
ATOM	304	N	TYR	A	23	-0.542	1.906	2.253	1.00	0.00	N
ATOM	305	CA	TYR	A	23	0.632	2.766	2.333	1.00	0.00	C
ATOM	306	C	TYR	A	23	0.475	3.975	1.414	1.00	0.00	C
ATOM	307	O	TYR	A	23	-0.119	3.879	0.340	1.00	0.00	O
ATOM	308	CB	TYR	A	23	1.891	1.978	1.958	1.00	0.00	C
ATOM	309	CG	TYR	A	23	3.143	2.827	1.888	1.00	0.00	C
ATOM	310	CD1	TYR	A	23	3.810	3.214	3.043	1.00	0.00	C
ATOM	311	CD2	TYR	A	23	3.656	3.238	0.664	1.00	0.00	C
ATOM	312	CE1	TYR	A	23	4.953	3.988	2.982	1.00	0.00	C
ATOM	313	CE2	TYR	A	23	4.799	4.012	0.595	1.00	0.00	C
ATOM	314	CZ	TYR	A	23	5.444	4.383	1.756	1.00	0.00	C
ATOM	315	OH	TYR	A	23	6.582	5.155	1.691	1.00	0.00	O
ATOM	316	H	TYR	A	23	-0.695	1.383	1.439	1.00	0.00	H
ATOM	317	HA	TYR	A	23	0.721	3.110	3.352	1.00	0.00	H
ATOM	318	1HB	TYR	A	23	2.056	1.207	2.695	1.00	0.00	H
ATOM	319	2HB	TYR	A	23	1.745	1.521	0.990	1.00	0.00	H
ATOM	320	HD1	TYR	A	23	3.423	2.901	4.001	1.00	0.00	H
ATOM	321	HD2	TYR	A	23	3.150	2.947	-0.244	1.00	0.00	H
ATOM	322	HE1	TYR	A	23	5.457	4.279	3.891	1.00	0.00	H
ATOM	323	HE2	TYR	A	23	5.184	4.323	-0.367	1.00	0.00	H
ATOM	324	HH	TYR	A	23	7.126	4.862	0.957	1.00	0.00	H
ATOM	325	N	CYS	A	24	1.016	5.111	1.846	1.00	0.00	N
ATOM	326	CA	CYS	A	24	0.939	6.340	1.066	1.00	0.00	C
ATOM	327	C	CYS	A	24	2.304	6.702	0.490	1.00	0.00	C
ATOM	328	O	CYS	A	24	3.292	6.786	1.220	1.00	0.00	O
ATOM	329	CB	CYS	A	24	0.418	7.487	1.932	1.00	0.00	C
ATOM	330	SG	CYS	A	24	-1.207	7.168	2.691	1.00	0.00	S
ATOM	331	H	CYS	A	24	1.476	5.122	2.711	1.00	0.00	H
ATOM	332	HA	CYS	A	24	0.249	6.173	0.251	1.00	0.00	H

ATOM	333	1HB	CYS	A	24	1.121	7.673	2.730	1.00	0.00	H
ATOM	334	2HB	CYS	A	24	0.329	8.375	1.325	1.00	0.00	H
ATOM	335	N	ARG	A	25	2.356	6.913	-0.822	1.00	0.00	N
ATOM	336	CA	ARG	A	25	3.604	7.265	-1.486	1.00	0.00	C
ATOM	337	C	ARG	A	25	4.021	8.698	-1.170	1.00	0.00	C
ATOM	338	O	ARG	A	25	5.153	9.100	-1.439	1.00	0.00	O
ATOM	339	CB	ARG	A	25	3.483	7.070	-2.992	1.00	0.00	C
ATOM	340	CG	ARG	A	25	4.456	6.045	-3.554	1.00	0.00	C
ATOM	341	CD	ARG	A	25	5.307	6.628	-4.673	1.00	0.00	C
ATOM	342	NE	ARG	A	25	4.492	7.237	-5.725	1.00	0.00	N
ATOM	343	CZ	ARG	A	25	4.271	8.546	-5.838	1.00	0.00	C
ATOM	344	NH1	ARG	A	25	4.785	9.398	-4.957	1.00	0.00	N
ATOM	345	NH2	ARG	A	25	3.530	9.007	-6.836	1.00	0.00	N
ATOM	346	H	ARG	A	25	1.537	6.832	-1.354	1.00	0.00	H
ATOM	347	HA	ARG	A	25	4.361	6.607	-1.118	1.00	0.00	H
ATOM	348	1HB	ARG	A	25	2.482	6.744	-3.217	1.00	0.00	H
ATOM	349	2HB	ARG	A	25	3.664	8.013	-3.479	1.00	0.00	H
ATOM	350	1HG	ARG	A	25	5.108	5.711	-2.761	1.00	0.00	H
ATOM	351	2HG	ARG	A	25	3.896	5.206	-3.940	1.00	0.00	H
ATOM	352	1HD	ARG	A	25	5.964	7.376	-4.258	1.00	0.00	H
ATOM	353	2HD	ARG	A	25	5.899	5.834	-5.105	1.00	0.00	H
ATOM	354	HE	ARG	A	25	4.092	6.637	-6.388	1.00	0.00	H
ATOM	355	1HH1	ARG	A	25	5.340	9.063	-4.199	1.00	0.00	H
ATOM	356	2HH1	ARG	A	25	4.612	10.378	-5.054	1.00	0.00	H
ATOM	357	1HH2	ARG	A	25	3.138	8.372	-7.502	1.00	0.00	H
ATOM	358	2HH2	ARG	A	25	3.363	9.989	-6.923	1.00	0.00	H
ATOM	359	N	PHE	A	26	3.103	9.457	-0.593	1.00	0.00	N
ATOM	360	CA	PHE	A	26	3.368	10.838	-0.231	1.00	0.00	C
ATOM	361	C	PHE	A	26	2.610	11.196	1.043	1.00	0.00	C
ATOM	362	O	PHE	A	26	2.227	10.315	1.814	1.00	0.00	O
ATOM	363	CB	PHE	A	26	2.964	11.769	-1.380	1.00	0.00	C
ATOM	364	CG	PHE	A	26	4.046	12.730	-1.783	1.00	0.00	C
ATOM	365	CD1	PHE	A	26	4.284	13.002	-3.120	1.00	0.00	C
ATOM	366	CD2	PHE	A	26	4.824	13.363	-0.826	1.00	0.00	C
ATOM	367	CE1	PHE	A	26	5.279	13.884	-3.496	1.00	0.00	C
ATOM	368	CE2	PHE	A	26	5.820	14.246	-1.195	1.00	0.00	C
ATOM	369	CZ	PHE	A	26	6.047	14.508	-2.532	1.00	0.00	C
ATOM	370	H	PHE	A	26	2.225	9.077	-0.401	1.00	0.00	H

ATOM	371	HA	PHE	A	26	4.427	10.938	-0.049	1.00	0.00	H
ATOM	372	1HB	PHE	A	26	2.711	11.173	-2.243	1.00	0.00	H
ATOM	373	2HB	PHE	A	26	2.099	12.346	-1.083	1.00	0.00	H
ATOM	374	HD1	PHE	A	26	3.684	12.516	-3.875	1.00	0.00	H
ATOM	375	HD2	PHE	A	26	4.647	13.158	0.221	1.00	0.00	H
ATOM	376	HE1	PHE	A	26	5.457	14.087	-4.542	1.00	0.00	H
ATOM	377	HE2	PHE	A	26	6.419	14.732	-0.439	1.00	0.00	H
ATOM	378	HZ	PHE	A	26	6.825	15.198	-2.822	1.00	0.00	H
ATOM	379	N	PHE	A	27	2.389	12.484	1.258	1.00	0.00	N
ATOM	380	CA	PHE	A	27	1.674	12.953	2.425	1.00	0.00	C
ATOM	381	C	PHE	A	27	0.292	12.313	2.499	1.00	0.00	C
ATOM	382	O	PHE	A	27	0.004	11.530	3.404	1.00	0.00	O
ATOM	383	CB	PHE	A	27	1.552	14.479	2.388	1.00	0.00	C
ATOM	384	CG	PHE	A	27	2.188	15.144	1.197	1.00	0.00	C
ATOM	385	CD1	PHE	A	27	3.479	15.642	1.273	1.00	0.00	C
ATOM	386	CD2	PHE	A	27	1.495	15.270	0.004	1.00	0.00	C
ATOM	387	CE1	PHE	A	27	4.067	16.253	0.182	1.00	0.00	C
ATOM	388	CE2	PHE	A	27	2.077	15.878	-1.092	1.00	0.00	C
ATOM	389	CZ	PHE	A	27	3.364	16.372	-1.002	1.00	0.00	C
ATOM	390	H	PHE	A	27	2.704	13.137	0.616	1.00	0.00	H
ATOM	391	HA	PHE	A	27	2.240	12.666	3.299	1.00	0.00	H
ATOM	392	1HB	PHE	A	27	0.513	14.742	2.382	1.00	0.00	H
ATOM	393	2HB	PHE	A	27	2.015	14.880	3.269	1.00	0.00	H
ATOM	394	HD1	PHE	A	27	4.030	15.550	2.199	1.00	0.00	H
ATOM	395	HD2	PHE	A	27	0.489	14.884	-0.068	1.00	0.00	H
ATOM	396	HE1	PHE	A	27	5.073	16.637	0.254	1.00	0.00	H
ATOM	397	HE2	PHE	A	27	1.527	15.970	-2.015	1.00	0.00	H
ATOM	398	HZ	PHE	A	27	3.822	16.848	-1.857	1.00	0.00	H
ATOM	399	N	ASN	A	28	-0.555	12.648	1.532	1.00	0.00	N
ATOM	400	CA	ASN	A	28	-1.907	12.105	1.474	1.00	0.00	C
ATOM	401	C	ASN	A	28	-2.437	12.127	0.043	1.00	0.00	C
ATOM	402	O	ASN	A	28	-3.223	13.002	-0.322	1.00	0.00	O
ATOM	403	CB	ASN	A	28	-2.840	12.901	2.391	1.00	0.00	C
ATOM	404	CG	ASN	A	28	-2.653	14.399	2.249	1.00	0.00	C
ATOM	405	OD1	ASN	A	28	-1.863	15.009	2.970	1.00	0.00	O
ATOM	406	ND2	ASN	A	28	-3.381	15.001	1.317	1.00	0.00	N
ATOM	407	H	ASN	A	28	-0.262	13.273	0.836	1.00	0.00	H
ATOM	408	HA	ASN	A	28	-1.868	11.081	1.815	1.00	0.00	H

ATOM	409	1HB	ASN	A	28	-3.864	12.661	2.146	1.00	0.00	H
ATOM	410	2HB	ASN	A	28	-2.646	12.626	3.417	1.00	0.00	H
ATOM	411	1HD2	ASN	A	28	-3.990	14.452	0.779	1.00	0.00	H
ATOM	412	2HD2	ASN	A	28	-3.280	15.969	1.203	1.00	0.00	H
ATOM	413	N	ALA	A	29	-2.002	11.166	-0.766	1.00	0.00	N
ATOM	414	CA	ALA	A	29	-2.439	11.092	-2.155	1.00	0.00	C
ATOM	415	C	ALA	A	29	-2.410	9.657	-2.673	1.00	0.00	C
ATOM	416	O	ALA	A	29	-3.456	9.057	-2.917	1.00	0.00	O
ATOM	417	CB	ALA	A	29	-1.571	11.985	-3.028	1.00	0.00	C
ATOM	418	H	ALA	A	29	-1.370	10.496	-0.422	1.00	0.00	H
ATOM	419	HA	ALA	A	29	-3.453	11.460	-2.205	1.00	0.00	H
ATOM	420	1HB	ALA	A	29	-1.817	11.822	-4.068	1.00	0.00	H
ATOM	421	2HB	ALA	A	29	-0.530	11.747	-2.865	1.00	0.00	H
ATOM	422	3HB	ALA	A	29	-1.748	13.020	-2.773	1.00	0.00	H
ATOM	423	N	PHE	A	30	-1.210	9.116	-2.842	1.00	0.00	N
ATOM	424	CA	PHE	A	30	-1.053	7.753	-3.336	1.00	0.00	C
ATOM	425	C	PHE	A	30	-1.161	6.744	-2.196	1.00	0.00	C
ATOM	426	O	PHE	A	30	-0.212	6.017	-1.903	1.00	0.00	O
ATOM	427	CB	PHE	A	30	0.293	7.598	-4.048	1.00	0.00	C
ATOM	428	CG	PHE	A	30	0.239	7.930	-5.513	1.00	0.00	C
ATOM	429	CD1	PHE	A	30	-0.238	9.157	-5.942	1.00	0.00	C
ATOM	430	CD2	PHE	A	30	0.667	7.012	-6.459	1.00	0.00	C
ATOM	431	CE1	PHE	A	30	-0.288	9.465	-7.289	1.00	0.00	C
ATOM	432	CE2	PHE	A	30	0.621	7.315	-7.807	1.00	0.00	C
ATOM	433	CZ	PHE	A	30	0.142	8.542	-8.222	1.00	0.00	C
ATOM	434	H	PHE	A	30	-0.411	9.645	-2.633	1.00	0.00	H
ATOM	435	HA	PHE	A	30	-1.848	7.564	-4.042	1.00	0.00	H
ATOM	436	1HB	PHE	A	30	1.015	8.254	-3.586	1.00	0.00	H
ATOM	437	2HB	PHE	A	30	0.627	6.575	-3.950	1.00	0.00	H
ATOM	438	HD1	PHE	A	30	-0.575	9.880	-5.213	1.00	0.00	H
ATOM	439	HD2	PHE	A	30	1.041	6.052	-6.136	1.00	0.00	H
ATOM	440	HE1	PHE	A	30	-0.662	10.426	-7.610	1.00	0.00	H
ATOM	441	HE2	PHE	A	30	0.956	6.591	-8.535	1.00	0.00	H
ATOM	442	HZ	PHE	A	30	0.105	8.780	-9.276	1.00	0.00	H
ATOM	443	N	CYS	A	31	-2.327	6.707	-1.557	1.00	0.00	N
ATOM	444	CA	CYS	A	31	-2.563	5.790	-0.448	1.00	0.00	C
ATOM	445	C	CYS	A	31	-3.314	4.548	-0.921	1.00	0.00	C
ATOM	446	O	CYS	A	31	-4.529	4.582	-1.107	1.00	0.00	O

ATOM	447	CB	CYS	A	31	-3.354	6.487	0.658	1.00	0.00	C
ATOM	448	SG	CYS	A	31	-2.525	7.950	1.359	1.00	0.00	S
ATOM	449	H	CYS	A	31	-3.045	7.312	-1.839	1.00	0.00	H
ATOM	450	HA	CYS	A	31	-1.602	5.488	-0.058	1.00	0.00	H
ATOM	451	1HB	CYS	A	31	-4.306	6.809	0.261	1.00	0.00	H
ATOM	452	2HB	CYS	A	31	-3.525	5.788	1.463	1.00	0.00	H
ATOM	453	N	TYR	A	32	-2.581	3.455	-1.109	1.00	0.00	N
ATOM	454	CA	TYR	A	32	-3.182	2.203	-1.560	1.00	0.00	C
ATOM	455	C	TYR	A	32	-2.630	1.021	-0.770	1.00	0.00	C
ATOM	456	O	TYR	A	32	-1.607	1.136	-0.097	1.00	0.00	O
ATOM	457	CB	TYR	A	32	-2.933	1.994	-3.056	1.00	0.00	C
ATOM	458	CG	TYR	A	32	-1.568	2.454	-3.520	1.00	0.00	C
ATOM	459	CD1	TYR	A	32	-0.413	2.005	-2.892	1.00	0.00	C
ATOM	460	CD2	TYR	A	32	-1.437	3.336	-4.584	1.00	0.00	C
ATOM	461	CE1	TYR	A	32	0.837	2.423	-3.314	1.00	0.00	C
ATOM	462	CE2	TYR	A	32	-0.192	3.757	-5.011	1.00	0.00	C
ATOM	463	CZ	TYR	A	32	0.940	3.299	-4.374	1.00	0.00	C
ATOM	464	OH	TYR	A	32	2.182	3.717	-4.797	1.00	0.00	O
ATOM	465	H	TYR	A	32	-1.616	3.491	-0.942	1.00	0.00	H
ATOM	466	HA	TYR	A	32	-4.246	2.268	-1.389	1.00	0.00	H
ATOM	467	1HB	TYR	A	32	-3.022	0.942	-3.285	1.00	0.00	H
ATOM	468	2HB	TYR	A	32	-3.676	2.542	-3.616	1.00	0.00	H
ATOM	469	HD1	TYR	A	32	-0.497	1.318	-2.062	1.00	0.00	H
ATOM	470	HD2	TYR	A	32	-2.326	3.694	-5.082	1.00	0.00	H
ATOM	471	HE1	TYR	A	32	1.723	2.062	-2.814	1.00	0.00	H
ATOM	472	HE2	TYR	A	32	-0.112	4.445	-5.842	1.00	0.00	H
ATOM	473	HH	TYR	A	32	2.193	4.675	-4.859	1.00	0.00	H
ATOM	474	N	CYS	A	33	-3.317	-0.116	-0.854	1.00	0.00	N
ATOM	475	CA	CYS	A	33	-2.895	-1.319	-0.141	1.00	0.00	C
ATOM	476	C	CYS	A	33	-1.444	-1.671	-0.460	1.00	0.00	C
ATOM	477	O	CYS	A	33	-0.976	-1.477	-1.582	1.00	0.00	O
ATOM	478	CB	CYS	A	33	-3.810	-2.497	-0.489	1.00	0.00	C
ATOM	479	SG	CYS	A	33	-3.186	-4.122	0.064	1.00	0.00	S
ATOM	480	H	CYS	A	33	-4.127	-0.145	-1.406	1.00	0.00	H
ATOM	481	HA	CYS	A	33	-2.975	-1.118	0.918	1.00	0.00	H
ATOM	482	1HB	CYS	A	33	-4.773	-2.344	-0.029	1.00	0.00	H
ATOM	483	2HB	CYS	A	33	-3.933	-2.542	-1.562	1.00	0.00	H
ATOM	484	N	ARG	A	34	-0.747	-2.197	0.540	1.00	0.00	N

ATOM	485	CA	ARG	A	34	0.647	-2.591	0.386	1.00	0.00	C
ATOM	486	C	ARG	A	34	0.907	-3.912	1.103	1.00	0.00	C
ATOM	487	O	ARG	A	34	0.720	-4.018	2.316	1.00	0.00	O
ATOM	488	CB	ARG	A	34	1.573	-1.506	0.936	1.00	0.00	C
ATOM	489	CG	ARG	A	34	3.050	-1.838	0.796	1.00	0.00	C
ATOM	490	CD	ARG	A	34	3.891	-0.582	0.635	1.00	0.00	C
ATOM	491	NE	ARG	A	34	5.318	-0.855	0.790	1.00	0.00	N
ATOM	492	CZ	ARG	A	34	6.078	-1.392	-0.162	1.00	0.00	C
ATOM	493	NH1	ARG	A	34	5.551	-1.715	-1.337	1.00	0.00	N
ATOM	494	NH2	ARG	A	34	7.366	-1.606	0.062	1.00	0.00	N
ATOM	495	H	ARG	A	34	-1.183	-2.327	1.407	1.00	0.00	H
ATOM	496	HA	ARG	A	34	0.842	-2.721	-0.669	1.00	0.00	H
ATOM	497	1HB	ARG	A	34	1.382	-0.584	0.408	1.00	0.00	H
ATOM	498	2HB	ARG	A	34	1.357	-1.361	1.984	1.00	0.00	H
ATOM	499	1HG	ARG	A	34	3.375	-2.365	1.681	1.00	0.00	H
ATOM	500	2HG	ARG	A	34	3.187	-2.466	-0.072	1.00	0.00	H
ATOM	501	1HD	ARG	A	34	3.717	-0.172	-0.349	1.00	0.00	H
ATOM	502	2HD	ARG	A	34	3.587	0.137	1.381	1.00	0.00	H
ATOM	503	HE	ARG	A	34	5.731	-0.626	1.648	1.00	0.00	H
ATOM	504	1HH1	ARG	A	34	4.580	-1.557	-1.513	1.00	0.00	H
ATOM	505	2HH1	ARG	A	34	6.128	-2.118	-2.048	1.00	0.00	H
ATOM	506	1HH2	ARG	A	34	7.767	-1.363	0.946	1.00	0.00	H
ATOM	507	2HH2	ARG	A	34	7.937	-2.009	-0.653	1.00	0.00	H
ATOM	508	N	LYS	A	35	1.330	-4.919	0.345	1.00	0.00	N
ATOM	509	CA	LYS	A	35	1.609	-6.237	0.908	1.00	0.00	C
ATOM	510	C	LYS	A	35	3.021	-6.317	1.486	1.00	0.00	C
ATOM	511	O	LYS	A	35	3.507	-7.404	1.797	1.00	0.00	O
ATOM	512	CB	LYS	A	35	1.423	-7.316	-0.160	1.00	0.00	C
ATOM	513	CG	LYS	A	35	0.015	-7.886	-0.206	1.00	0.00	C
ATOM	514	CD	LYS	A	35	-0.268	-8.568	-1.535	1.00	0.00	C
ATOM	515	CE	LYS	A	35	0.516	-9.866	-1.674	1.00	0.00	C
ATOM	516	NZ	LYS	A	35	-0.380	-11.052	-1.750	1.00	0.00	N
ATOM	517	H	LYS	A	35	1.454	-4.775	-0.617	1.00	0.00	H
ATOM	518	HA	LYS	A	35	0.899	-6.410	1.703	1.00	0.00	H
ATOM	519	1HB	LYS	A	35	1.649	-6.893	-1.127	1.00	0.00	H
ATOM	520	2HB	LYS	A	35	2.110	-8.125	0.039	1.00	0.00	H
ATOM	521	1HG	LYS	A	35	-0.098	-8.608	0.589	1.00	0.00	H
ATOM	522	2HG	LYS	A	35	-0.694	-7.082	-0.067	1.00	0.00	H

ATOM	523	1HD	LYS	A	35	-1.322	-8.790	-1.598	1.00	0.00	H
ATOM	524	2HD	LYS	A	35	0.012	-7.902	-2.338	1.00	0.00	H
ATOM	525	1HE	LYS	A	35	1.109	-9.817	-2.576	1.00	0.00	H
ATOM	526	2HE	LYS	A	35	1.169	-9.973	-0.820	1.00	0.00	H
ATOM	527	1HZ	LYS	A	35	-0.019	-11.814	-1.140	1.00	0.00	H
ATOM	528	2HZ	LYS	A	35	-0.430	-11.401	-2.728	1.00	0.00	H
ATOM	529	3HZ	LYS	A	35	-1.340	-10.797	-1.436	1.00	0.00	H
ATOM	530	N	LEU	A	36	3.679	-5.167	1.633	1.00	0.00	N
ATOM	531	CA	LEU	A	36	5.025	-5.128	2.175	1.00	0.00	C
ATOM	532	C	LEU	A	36	5.990	-5.915	1.305	1.00	0.00	C
ATOM	533	O	LEU	A	36	5.918	-7.141	1.223	1.00	0.00	O
ATOM	534	CB	LEU	A	36	5.039	-5.664	3.604	1.00	0.00	C
ATOM	535	CG	LEU	A	36	4.725	-4.611	4.663	1.00	0.00	C
ATOM	536	CD1	LEU	A	36	3.342	-4.840	5.256	1.00	0.00	C
ATOM	537	CD2	LEU	A	36	5.783	-4.613	5.756	1.00	0.00	C
ATOM	538	H	LEU	A	36	3.251	-4.329	1.374	1.00	0.00	H
ATOM	539	HA	LEU	A	36	5.339	-4.096	2.190	1.00	0.00	H
ATOM	540	1HB	LEU	A	36	4.308	-6.458	3.678	1.00	0.00	H
ATOM	541	2HB	LEU	A	36	6.017	-6.072	3.807	1.00	0.00	H
ATOM	542	HG	LEU	A	36	4.730	-3.640	4.190	1.00	0.00	H
ATOM	543	1HD1	LEU	A	36	2.594	-4.694	4.490	1.00	0.00	H
ATOM	544	2HD1	LEU	A	36	3.176	-4.138	6.060	1.00	0.00	H
ATOM	545	3HD1	LEU	A	36	3.276	-5.848	5.638	1.00	0.00	H
ATOM	546	1HD2	LEU	A	36	5.340	-4.294	6.687	1.00	0.00	H
ATOM	547	2HD2	LEU	A	36	6.581	-3.934	5.486	1.00	0.00	H
ATOM	548	3HD2	LEU	A	36	6.183	-5.609	5.870	1.00	0.00	H
ATOM	549	N	GLY	A	37	6.890	-5.193	0.658	1.00	0.00	N
ATOM	550	CA	GLY	A	37	7.871	-5.823	-0.206	1.00	0.00	C
ATOM	551	C	GLY	A	37	9.003	-6.472	0.567	1.00	0.00	C
ATOM	552	O	GLY	A	37	10.174	-6.191	0.315	1.00	0.00	O
ATOM	553	H	GLY	A	37	6.888	-4.219	0.770	1.00	0.00	H
ATOM	554	1HA	GLY	A	37	7.376	-6.578	-0.800	1.00	0.00	H
ATOM	555	2HA	GLY	A	37	8.284	-5.076	-0.868	1.00	0.00	H
ATOM	556	N	THR	A	38	8.654	-7.347	1.506	1.00	0.00	N
ATOM	557	CA	THR	A	38	9.649	-8.042	2.312	1.00	0.00	C
ATOM	558	C	THR	A	38	10.046	-9.357	1.655	1.00	0.00	C
ATOM	559	O	THR	A	38	9.603	-10.429	2.070	1.00	0.00	O
ATOM	560	CB	THR	A	38	9.110	-8.298	3.720	1.00	0.00	C

ATOM	561	OG1	THR	A	38	7.895	-9.024	3.669	1.00	0.00	O
ATOM	562	CG2	THR	A	38	8.854	-7.029	4.505	1.00	0.00	C
ATOM	563	H	THR	A	38	7.707	-7.534	1.658	1.00	0.00	H
ATOM	564	HA	THR	A	38	10.522	-7.408	2.379	1.00	0.00	H
ATOM	565	HB	THR	A	38	9.832	-8.885	4.269	1.00	0.00	H
ATOM	566	HG1	THR	A	38	7.196	-8.459	3.332	1.00	0.00	H
ATOM	567	1HG2	THR	A	38	8.717	-6.205	3.820	1.00	0.00	H
ATOM	568	2HG2	THR	A	38	9.698	-6.827	5.147	1.00	0.00	H
ATOM	569	3HG2	THR	A	38	7.965	-7.150	5.106	1.00	0.00	H
ATOM	570	N	ALA	A	39	10.878	-9.267	0.625	1.00	0.00	N
ATOM	571	CA	ALA	A	39	11.335	-10.449	-0.099	1.00	0.00	C
ATOM	572	C	ALA	A	39	12.107	-11.402	0.810	1.00	0.00	C
ATOM	573	O	ALA	A	39	12.308	-12.568	0.469	1.00	0.00	O
ATOM	574	CB	ALA	A	39	12.191	-10.040	-1.288	1.00	0.00	C
ATOM	575	H	ALA	A	39	11.190	-8.381	0.342	1.00	0.00	H
ATOM	576	HA	ALA	A	39	10.464	-10.959	-0.474	1.00	0.00	H
ATOM	577	1HB	ALA	A	39	11.591	-9.475	-1.985	1.00	0.00	H
ATOM	578	2HB	ALA	A	39	12.577	-10.922	-1.775	1.00	0.00	H
ATOM	579	3HB	ALA	A	39	13.014	-9.429	-0.946	1.00	0.00	H
ATOM	580	N	MET	A	40	12.536	-10.904	1.963	1.00	0.00	N
ATOM	581	CA	MET	A	40	13.283	-11.718	2.914	1.00	0.00	C
ATOM	582	C	MET	A	40	12.334	-12.525	3.796	1.00	0.00	C
ATOM	583	O	MET	A	40	12.618	-13.671	4.143	1.00	0.00	O
ATOM	584	CB	MET	A	40	14.178	-10.833	3.783	1.00	0.00	C
ATOM	585	CG	MET	A	40	15.537	-11.448	4.081	1.00	0.00	C
ATOM	586	SD	MET	A	40	16.877	-10.242	4.016	1.00	0.00	S
ATOM	587	CE	MET	A	40	16.815	-9.759	2.292	1.00	0.00	C
ATOM	588	H	MET	A	40	12.347	-9.970	2.179	1.00	0.00	H
ATOM	589	HA	MET	A	40	13.902	-12.400	2.352	1.00	0.00	H
ATOM	590	1HB	MET	A	40	14.336	-9.892	3.277	1.00	0.00	H
ATOM	591	2HB	MET	A	40	13.679	-10.645	4.722	1.00	0.00	H
ATOM	592	1HG	MET	A	40	15.512	-11.882	5.069	1.00	0.00	H
ATOM	593	2HG	MET	A	40	15.733	-12.222	3.353	1.00	0.00	H
ATOM	594	1HE	MET	A	40	16.197	-8.880	2.186	1.00	0.00	H
ATOM	595	2HE	MET	A	40	16.398	-10.566	1.708	1.00	0.00	H
ATOM	596	3HE	MET	A	40	17.814	-9.541	1.943	1.00	0.00	H
ATOM	597	N	ASN	A	41	11.206	-11.920	4.149	1.00	0.00	N
ATOM	598	CA	ASN	A	41	10.211	-12.581	4.986	1.00	0.00	C

ATOM	599	C	ASN	A	41	8.806	-12.072	4.664	1.00	0.00	C
ATOM	600	O	ASN	A	41	8.174	-11.402	5.481	1.00	0.00	O
ATOM	601	CB	ASN	A	41	10.529	-12.356	6.467	1.00	0.00	C
ATOM	602	CG	ASN	A	41	11.147	-13.578	7.117	1.00	0.00	C
ATOM	603	OD1	ASN	A	41	11.058	-14.688	6.591	1.00	0.00	O
ATOM	604	ND2	ASN	A	41	11.780	-13.382	8.268	1.00	0.00	N
ATOM	605	H	ASN	A	41	11.035	-11.007	3.837	1.00	0.00	H
ATOM	606	HA	ASN	A	41	10.253	-13.639	4.775	1.00	0.00	H
ATOM	607	1HB	ASN	A	41	11.223	-11.534	6.559	1.00	0.00	H
ATOM	608	2HB	ASN	A	41	9.618	-12.114	6.994	1.00	0.00	H
ATOM	609	1HD2	ASN	A	41	11.812	-12.471	8.629	1.00	0.00	H
ATOM	610	2HD2	ASN	A	41	12.189	-14.156	8.710	1.00	0.00	H
ATOM	611	N	PRO	A	42	8.299	-12.384	3.459	1.00	0.00	N
ATOM	612	CA	PRO	A	42	6.966	-11.956	3.028	1.00	0.00	C
ATOM	613	C	PRO	A	42	5.855	-12.784	3.665	1.00	0.00	C
ATOM	614	O	PRO	A	42	6.027	-13.974	3.927	1.00	0.00	O
ATOM	615	CB	PRO	A	42	7.002	-12.182	1.518	1.00	0.00	C
ATOM	616	CG	PRO	A	42	7.954	-13.313	1.332	1.00	0.00	C
ATOM	617	CD	PRO	A	42	8.987	-13.177	2.420	1.00	0.00	C
ATOM	618	HA	PRO	A	42	6.800	-10.909	3.235	1.00	0.00	H
ATOM	619	1HB	PRO	A	42	6.013	-12.435	1.165	1.00	0.00	H
ATOM	620	2HB	PRO	A	42	7.351	-11.289	1.023	1.00	0.00	H
ATOM	621	1HG	PRO	A	42	7.431	-14.252	1.428	1.00	0.00	H
ATOM	622	2HG	PRO	A	42	8.422	-13.243	0.362	1.00	0.00	H
ATOM	623	1HD	PRO	A	42	9.265	-14.149	2.800	1.00	0.00	H
ATOM	624	2HD	PRO	A	42	9.857	-12.655	2.051	1.00	0.00	H
ATOM	625	N	CYS	A	43	4.715	-12.147	3.909	1.00	0.00	N
ATOM	626	CA	CYS	A	43	3.582	-12.799	4.505	1.00	0.00	C
ATOM	627	C	CYS	A	43	2.788	-13.563	3.458	1.00	0.00	C
ATOM	628	O	CYS	A	43	3.287	-14.505	2.841	1.00	0.00	O
ATOM	629	CB	CYS	A	43	2.717	-11.741	5.173	1.00	0.00	C
ATOM	630	SG	CYS	A	43	3.620	-10.655	6.325	1.00	0.00	S
ATOM	631	H	CYS	A	43	4.627	-11.204	3.684	1.00	0.00	H
ATOM	632	HA	CYS	A	43	3.923	-13.485	5.246	1.00	0.00	H
ATOM	633	1HB	CYS	A	43	2.274	-11.119	4.409	1.00	0.00	H
ATOM	634	2HB	CYS	A	43	1.942	-12.227	5.718	1.00	0.00	H
ATOM	635	N	SER	A	44	1.554	-13.151	3.270	1.00	0.00	N
ATOM	636	CA	SER	A	44	0.670	-13.781	2.308	1.00	0.00	C

ATOM	637	C	SER	A	44	0.936	-13.263	0.900	1.00	0.00	C
ATOM	638	O	SER	A	44	0.285	-12.326	0.439	1.00	0.00	O
ATOM	639	CB	SER	A	44	-0.791	-13.539	2.688	1.00	0.00	C
ATOM	640	OG	SER	A	44	-1.145	-14.269	3.851	1.00	0.00	O
ATOM	641	H	SER	A	44	1.231	-12.404	3.801	1.00	0.00	H
ATOM	642	HA	SER	A	44	0.869	-14.835	2.337	1.00	0.00	H
ATOM	643	1HB	SER	A	44	-0.943	-12.488	2.879	1.00	0.00	H
ATOM	644	2HB	SER	A	44	-1.429	-13.852	1.874	1.00	0.00	H
ATOM	645	HG	SER	A	44	-1.908	-14.821	3.663	1.00	0.00	H
ATOM	646	N	ARG	A	45	1.898	-13.880	0.219	1.00	0.00	N
ATOM	647	CA	ARG	A	45	2.250	-13.481	-1.138	1.00	0.00	C
ATOM	648	C	ARG	A	45	2.882	-14.639	-1.904	1.00	0.00	C
ATOM	649	O	ARG	A	45	3.804	-14.443	-2.697	1.00	0.00	O
ATOM	650	CB	ARG	A	45	3.211	-12.291	-1.108	1.00	0.00	C
ATOM	651	CG	ARG	A	45	3.124	-11.405	-2.341	1.00	0.00	C
ATOM	652	CD	ARG	A	45	4.407	-11.447	-3.153	1.00	0.00	C
ATOM	653	NE	ARG	A	45	5.470	-10.655	-2.540	1.00	0.00	N
ATOM	654	CZ	ARG	A	45	6.607	-10.337	-3.156	1.00	0.00	C
ATOM	655	NH1	ARG	A	45	6.833	-10.743	-4.399	1.00	0.00	N
ATOM	656	NH2	ARG	A	45	7.520	-9.611	-2.526	1.00	0.00	N
ATOM	657	H	ARG	A	45	2.382	-14.621	0.639	1.00	0.00	H
ATOM	658	HA	ARG	A	45	1.343	-13.184	-1.643	1.00	0.00	H
ATOM	659	1HB	ARG	A	45	2.988	-11.685	-0.241	1.00	0.00	H
ATOM	660	2HB	ARG	A	45	4.222	-12.661	-1.027	1.00	0.00	H
ATOM	661	1HG	ARG	A	45	2.307	-11.746	-2.960	1.00	0.00	H
ATOM	662	2HG	ARG	A	45	2.939	-10.387	-2.028	1.00	0.00	H
ATOM	663	1HD	ARG	A	45	4.737	-12.473	-3.230	1.00	0.00	H
ATOM	664	2HD	ARG	A	45	4.206	-11.061	-4.142	1.00	0.00	H
ATOM	665	HE	ARG	A	45	5.330	-10.342	-1.622	1.00	0.00	H
ATOM	666	1HH1	ARG	A	45	6.148	-11.291	-4.880	1.00	0.00	H
ATOM	667	2HH1	ARG	A	45	7.688	-10.501	-4.856	1.00	0.00	H
ATOM	668	1HH2	ARG	A	45	7.356	-9.303	-1.590	1.00	0.00	H
ATOM	669	2HH2	ARG	A	45	8.375	-9.373	-2.989	1.00	0.00	H
ATOM	670	N	THR	A	46	2.382	-15.846	-1.661	1.00	0.00	N
ATOM	671	CA	THR	A	46	2.899	-17.036	-2.328	1.00	0.00	C
ATOM	672	C	THR	A	46	2.368	-17.133	-3.754	1.00	0.00	C
ATOM	673	O	THR	A	46	2.909	-17.946	-4.533	1.00	0.00	O
ATOM	674	CB	THR	A	46	2.522	-18.293	-1.543	1.00	0.00	C

ATOM	675	OG1	THR	A	46	2.802	-18.127	-0.165	1.00	0.00	O
ATOM	676	CG2	THR	A	46	3.250	-19.534	-2.014	1.00	0.00	C
ATOM	677	OXT	THR	A	46	1.413	-16.397	-4.081	1.00	0.00	O
ATOM	678	H	THR	A	46	1.648	-15.940	-1.019	1.00	0.00	H
ATOM	679	HA	THR	A	46	3.976	-16.954	-2.363	1.00	0.00	H
ATOM	680	HB	THR	A	46	1.461	-18.470	-1.656	1.00	0.00	H
ATOM	681	HG1	THR	A	46	2.011	-17.825	0.289	1.00	0.00	H
ATOM	682	1HG2	THR	A	46	3.933	-19.271	-2.809	1.00	0.00	H
ATOM	683	2HG2	THR	A	46	2.534	-20.255	-2.378	1.00	0.00	H
ATOM	684	3HG2	THR	A	46	3.803	-19.960	-1.191	1.00	0.00	H
TER	685		THR	A	46						

Example 3

Screening of mini-AGRPs for melanocortin Receptor activity.

The mini-AGRP, given by the sequence: Ac-CVRLHESCLGQQVPCCDPAATCYCRFFNAFCYCR-NH_2 (SEQ ID NO:5) was synthesized using Fmoc solid phase chemistry (where Ac is an acetyl group and -NH_2 denotes a C-terminal amide). The peptide was prepared as a single chain and then partially purified by HPLC. The peptide was then folded under oxidizing conditions to form the disulfide bonds, and then repurified. HPLC indicated a uniform product and mass spectrometry demonstrated loss of 8.0 AMUs consistent with formation of four disulfide bonds. The folding yield was greater than 80% as determined by HPLC.

Amino acid analysis verified the correct omposition and allowed for the preparation of stock solutions of known mini-AGRP concentration (to within 5%).

The mini-AGRP binding was tested by examining its ability to displace NDP-MSH, a well established high affinity MCR agonist. To within experimental error, the mini-AGRP displaced NDP-MSH at each of the melanocortin receptors (MC1R, MC3R, MC4R and MC5R) with potency equivalent to that of AGRP(87-132) (MARP). Antagonist activity was then determined using a cAMP assay. With both brain receptors MC3R and MC4R, mini-AGRP exhibited potent activity as determined by its ability to suppress α-MSH stimulated cAMP production. Taken together, these studies demonstrate that this mini-AGRP is readily synthesized, folded to a uniform product, and exhibits MCR binding,

selectivity and antagonist activity comparable to that of the AGRP C-terminal domain (AGRP(87-132); MARP).

Example 4

Synthesis and Assay of Melanocortin Receptor Binding Ligands (Peptidomimetics)

A) Synthesis of non-peptide Compounds

A synthesis scheme for the peptidomimetics is illustrated in Figure 4. To 20% Piperidine/DMF 2 x 5min treated and DMF washed Rink Amide-MBHA resin (Novabiochem, Switzerland), 5 equivalents of Fmoc-Aminoisobutyric acid (Fmoc-Aib) or 7 of Bromoacetic acid (Aldrich, USA) in DMF, activated as the symmetric anhydride by incubating 30 min. with Diisopropylcarbodiimide (DIC), is reacted for 20 min., then washed with DMF. The washed resin, in the case of Fmoc-Aib treated with 20% Piperidine/DMF 2 x 5 min. and DMF washed, is reacted with 20-40 equivalents, concentration 2-4 M, of primary or secondary amine or bromide of choice in DMSO or DMF with the appropriate tertiary amine (note: methylamine is only available as a 2M solution in various solvents). This is known as the submonomer approach (Zuckermann et al., *J Amer Chem Soc* 1992).

The process is repeated 1-2 times, until yielding a complete molecule, or intermediate. To the intermediate, 7 equivalents of bromoacetic acid or 5-8 equivalents of a carboxylic acid of choice, activated as the symmetric anhydride by incubating 30 min. with Diisopropylcarbodiimide (DIC) or as the assymetric anhydride, in the case of an enantiomeric carboxylic acid, is reacted for 0.5-2 hr., then washed with DMF. If the intermidiate is brominated then reacted with 20 equivalents of the thiol or 2-4 M of the primary or secondary amine of choice, if the thiol or amine contains one or more accessible amine then the carboxylic acid of choice is coupled or the amine is guanylated. If the intermediate has an accessible amine then one of the following may happen: 1) alkylation by the primary or secondary bromide of choice 2) guanylation 3) a carboxylic acid of choice is coupled. The reaction of amines with 3,5-Dimethylpyrazole-1-carboxamidine nitrate or O-methylisourea is well known in the art. The resulting non-peptides are cleaved, usually, but not limited to, by exposing the resin bound molecules to 95:5 TFA:H_2O. The cleavage from Resin is done in 95:2.5:2.5 TFA:TIS:water.

B) Activity Assay

HEK 293 cells stably expressing the human melanocortin receptors (hMCR's) 1-5 are grown in media. After removal from media, cells are washed twice with MEM (Life Technologies, Gaithersberg, MD) and then preincubated with AGRP (87-132) in 0.5 ml MEM 0.2% BSA for 30 min. before incubation with ^{125}I-NDP-MSH or 125 I-AGRP (87-132). Concentrations of cold ligand are added from 100 mM - 1 nM. Binding reactions are terminated by washing cells twice with MEM 0.2% BSA. The cells are lysed with .1 N NaOH 1% Triton X, and radioactivity of the lysate is quantified with a gamma-analytical counter. AGRP (87-132) hMC4R $IC_{50} = 9$ nM ± 1.7 nM. $IC_{50} < 100$ mM is considered a specific binder

It is apparent from the above results and discussion that the subject invention provides an important new class of MCR ligands, where the ligands have broad ranging applicability as either MCR antagonists and agonists and therefore find use in a variety of therapeutic and related applications.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

CLAIMS

What is claimed is:

1. A method of modulating the activity of a melanocortin receptor, said method comprising contacting said receptor with a peptide having the formula:

$CX^1X^2X^3X^4X^5X^6CX^7X^8X^9X^{10}X^{11}X^{12}CCDPX^{13}ATCYCX^{14}X^{15}X^{16}NAFCYCR_n$

wherein

X^1, X^2, X^3, X^4, X^5, X^6, X^7, X^8, X^9, X^{10}, X^{11}, X^{12}, X^{13}, X^{14}, X^{15}, and X^{16} are independently selected amino acids, and

n is zero or one.

2. The method of claim 1, wherein X^1, X^2, X^3, X^4, X^5, X^6, X^7, X^8, X^9, X^{10}, X^{11}, X^{12}, X^{13}, X^{14}, X^{15}, and X^{16} are independently selected from the group consisting of alanine, asparagine, arginine,aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine.

3. The method of claim 1, wherein said peptide is not CVRLHESCLGQQVPCCDPAATCYCRFFNAFCYC (SEQ ID NO:3).

4. The method of claim 1, wherein $X^1X^2X^3X^4X^5X^6$ is VRLHES or conservative substitutions thereof.

5. The method of claim 4, wherein $X^1X^2X^3X^4X^5X^6$ is VRLHES.

6. The method of claim 1, wherein $X^7X^8X^9X^{10}X^{11}X^{12}$ is LGQQVP or conservative substitutions thereof.

7. The method of claim 6, wherein $X^7X^8X^9X^{10}X^{11}X^{12}$ is LGQQVP.

8. The method of claim 7, wherein $X^7X^8X^9X^{10}X^{11}X^{12}$ is LGQQVP or conservative substitutions thereof.

9. The method of claim 8, wherein $X^7X^8X^9X^{10}X^{11}X^{12}$ is LGQQVP.

10. The method of claim 1, wherein X^{13} is not a cysteine.

11. The method of claim 1, wherein X^{13} is A.

12. The method of claim 1, wherein $X^{14}X^{15}X^{16}$ is RFF or conservative substitutions thereof.

13. The method of claim 4, wherein $X^{14}X^{15}X^{16}$ is RFF or conservative substitutions thereof.

14. The method of claim 8, wherein $X^{14}X^{15}X^{16}$ is RFF or conservative substitutions thereof.

15. The method of claim 1, wherein said receptor is in a cell culture.

16. The method of claim 1, wherein said receptor is *in vivo* culture.

17. The method of claim 1, wherein said receptor is an MC3 receptor.

18. The method of claim 1, wherein said receptor is an MC4 receptor.

19. A library for screening for modulators of a melanocortin receptor, said library comprising a plurality of polypeptide members wherein said members have the formula:

$$CX^1X^2X^3X^4X^5X^6CX^7X^8X^9X^{10}X^{11}X^{12}CCDPX^{13}ATCYCX^{14}X^{15}X^{16}NAFCYCR_n$$

wherein

X^1, X^2, X^3, X^4, X^5, X^6, X^7, X^8, X^9, X^{10}, X^{11}, X^{12}, X^{13}, X^{14}, X^{15}, and X^{16} are independently selected amino acids, and

n is zero or one.

20. The library of claim 19, wherein X^1, X^2, X^3, X^4, X^5, X^6, X^7, X^8, X^9, X^{10}, X^{11}, X^{12}, X^{13}, X^{14}, X^{15}, and X^{16} are independently selected from the group consisting of aspartic acid, alanine, asparagine, arginine,cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine.

21. The method of claim 19, wherein $X^1X^2X^3X^4X^5X^6$ is VRLHES or conservative substitutions thereof.

22. The method of claim 21, wherein $X^1X^2X^3X^4X^5X^6$ is VRLHES.

23. The method of claim 19, wherein $X^7X^8X^9X^{10}X^{11}X^{12}$ is LGQQVP or conservative substitutions thereof.

24. The method of claim 23, wherein $X^7X^8X^9X^{10}X^{11}X^{12}$ is LGQQVP.

25. The method of claim 24, wherein $X^7X^8X^9X^{10}X^{11}X^{12}$ is LGQQVP or conservative substitutions thereof.

26. The method of claim 25, wherein $X^7X^8X^9X^{10}X^{11}X^{12}$ is LGQQVP.

27. The method of claim 19, wherein X^{13} is not a cysteine.

28. The method of claim 19, wherein X^{13} is A.

29. The method of claim 19, wherein $X^{14}X^{15}X^{16}$ is RFF or conservative substitutions thereof.

30. The method of claim 21, wherein $X^{14}X^{15}X^{16}$ is RFF or conservative substitutions thereof.

31. The method of claim 25, wherein $X^{14}X^{15}X^{16}$ is RFF or conservative substitutions thereof.

32. A method of prescreening for a modulator of a melanocortin receptor, said method comprising:

i) contacting a melanocortin receptor a peptide having the formula:

$$CX^1X^2X^3X^4X^5X^6CX^7X^8X^9X^{10}X^{11}X^{12}CCDPX^{13}ATCYCX^{14}X^{15}X^{16}NAFCYCR_n$$

wherein

X^1, X^2, X^3, X^4, X^5, X^6, X^7, X^8, X^9, X^{10}, X^{11}, X^{12}, X^{13}, X^{14}, X^{15}, and X^{16} are independently selected amino acids, and

n is zero or one; and

ii) detecting binding of said peptide to said melanocortin receptor wherein specific binding of said peptide to said melanocortin receptor indicates that said peptide is a potential modulator of said melanocortin receptor.

33. The method of claim 32, wherein said peptide is not CVRLHESCLGQQVPCCDPAATCYCRFFNAFCYC (SEQ ID NO:3).

34. The method of claim 32, wherein said melanocortin receptor is selected from the group consisting of MC3r, and MC4r.

35. A method of screening for a modulator of melanocortin receptor activity, said method comprising:

i) contacting a melanocortin receptor with a peptide having the formula:

$CX^1X^2X^3X^4X^5X^6CX^7X^8X^9X^{10}X^{11}X^{12}CCDPX^{13}ATCYCX^{14}X^{15}X^{16}NAFCYCR_n$

wherein

X^1, X^2, X^3, X^4, X^5, X^6, X^7, X^8, X^9, X^{10}, X^{11}, X^{12}, X^{13}, X^{14}, X^{15}, and X^{16} are independently selected amino acids, and

n is zero or one; and

ii) detecting activity of said melanocortin receptor wherein a difference in activity of said receptor, as compared to a control, indicates that said peptide is a modulator of melanocortin receptor activity.

36. The method of claim 35, wherein said control is a negative control comprising the same assay without said peptide.

37. The method of claim 35, wherein said peptide is not CVRLHESCLGQQVPCCDPAATCYCRFFNAFCYC (SEQ ID NO:3).

38. The method of claim 35, wherein said melanocortin receptor is selected from the group consisting of MC3r, and MC4r.

39. A polypeptide comprising a peptide sequence having the formula 35wherein said polypeptide is not AGRP and said polypeptide is not MARP.

40. The polypeptide of claim 39, wherein said polypeptide excludes one or more of the final 13 residues of MARP (residues 34-46 of MARP).

41. The polypeptide of claim 39, wherein said polyypeptide has the formula:

$CX^1X^2X^3X^4X^5X^6CX^7X^8X^9X^{10}X^{11}X^{12}CCDPX^{13}ATCYCX^{14}X^{15}X^{16}NAFCYCR_n$

wherein

X^1, X^2, X^3, X^4, X^5, X^6, X^7, X^8, X^9, X^{10}, X^{11}, X^{12}, X^{13}, X^{14}, X^{15}, and X^{16} are independently selected amino acids, and

n is zero or one.

42. The polypeptide of claim 41, wherein said polypeptide is not CVRLHESCLGQQVPCCDPAATCYCRFFNAFCYC (SEQ ID NO:3).

43. A pharmaceutical composition comprising:

a pharmaceutically acceptable excipient; and

a polypeptide having the formula:

$CX^1X^2X^3X^4X^5X^6CX^7X^8X^9X^{10}X^{11}X^{12}CCDPX^{13}ATCYCX^{14}X^{15}X^{16}NAFCYCR_n$

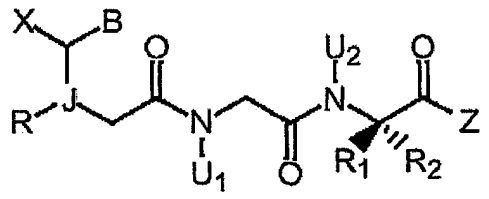
wherein

X^1, X^2, X^3, X^4, X^5, X^6, X^7, X^8, X^9, X^{10}, X^{11}, X^{12}, X^{13}, X^{14}, X^{15}, and X^{16} are independently selected amino acids, and

n is zero or one.

44. The composition of claim 43, wherein said polypeptide is not CVRLHESCLGQQVPCCDPAATCYCRFFNAFCYC (SEQ ID NO:3).

45. A non-peptide melanocortin receptor ligand of the structural formula:



wherein

B, U_1, U_2, R, R_1, and R_2 are independently selected from the group consisting of: hydrogen, alkyl, derivatized alkyl, cycloalkyl, derivatized cycloalkyl, halocycloalkyl, aloxycycloalkyl, heterocycloalkyl, aryl, arylalkyl, heteroaryl, and heteroarylalkyl;

J is selected from the group consisting of carbon, nitrogen, silicon, and sulfur;

X is selected from the group consisting of hydrogen, carbon, nitrogen, oxygen, silicon, and sulfur; and

Z is selected from the group consisting of a continuing peptide bond, a hydroxyl; -NH_2-, -NH-(n), and -N-(n,n'), and -O-(y), where where n and n' are independently selected from the group consisting of alkyl, alkenyl, alkynyl, cycloalkyl, aryl, arylalkyl, heteroaryl, heteroarylalkyl, and a derivatized form thereof, and y is selected from the group consisting of alkyl, alkenyl, alkynyl, cycloalkyl, aryl, arylalkyl, heteroaryl heteroarylalkyl, and a derivatized form thereof.

46. The non-peptide melanocortin receptor ligand according to claim 45, wherein said ligand is a ligand for a melanocortin receptor selected from the group consisting of MC3r and MC4r.

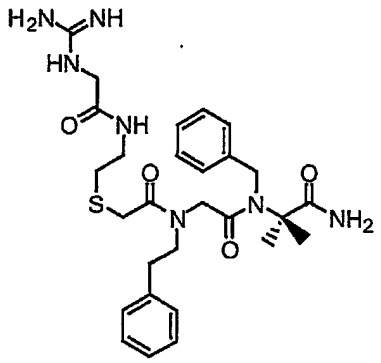
47. The non-peptide melanocortin receptor ligand according to claim 45, wherein said ligand has a molecular weight ranging from about 200 to 1000 daltons.

48. The non-peptide melanocortin receptor ligand according to claim 45, wherein said ligand has a structure that mimics the backbone of the AGRP active loop.

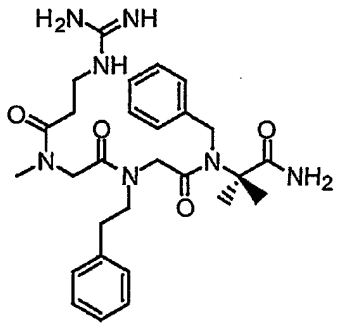
49. The non-peptide melanocortin receptor ligand according to claim 45, wherein said ligand comprises a terminal gaunidino moiety.

50. The non-peptide melanocortin receptor ligand according to claim 45, wherein said ligand comprises at least one methylbenzyl moiety.

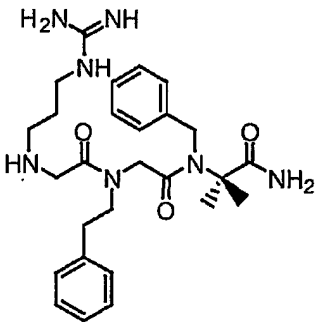
51. The non-peptide melanocortin receptor ligand according to claim 45, wherein said ligand has the structural formula:



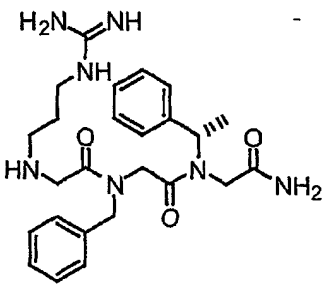
52. The non-peptide melanocortin receptor ligand according to claim 45, wherein said ligand has the structural formula:



53. The non-peptide melanocortin receptor ligand according to claim 45, wherein said ligand has the structural formula:



54. The non-peptide melanocortin receptor ligand according to claim 45, wherein said ligand has the structural formula:



55. A pharmaceutical preparation of a non-peptide melanocortin receptor ligand according to claim 45.

56. The pharmaceutical preparation according to claim 55, wherein said ligand is a melanocortin receptor antagonist.

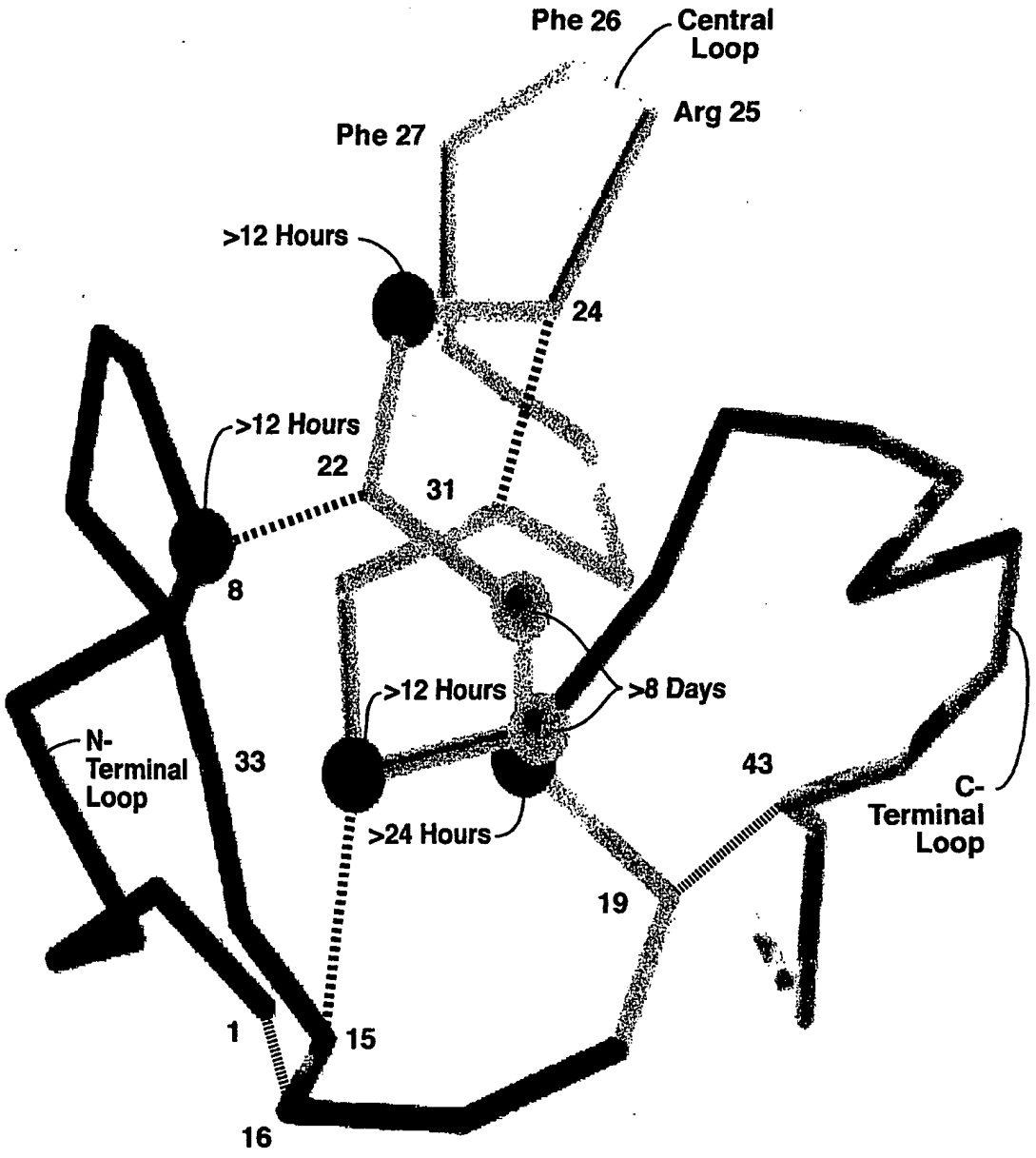
57. The pharmaceutical preparation according to claim 56, wherein said ligand is a melanocortin receptor agonist.

58. A method for modulating a melanocortin receptor mediated physiological process, said method comprising:

contacting said melanocortin receptor with a non-peptide melanocortin receptor ligand according to claim 45.

59. The method according to claim 58, wherein said ligand is a melanocortin receptor agonist.

60. The method according to claim 58, wherein said ligand is a melanocortin receptor antagonist.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22
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C-Terminal Loop
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15
16

FIG._1A

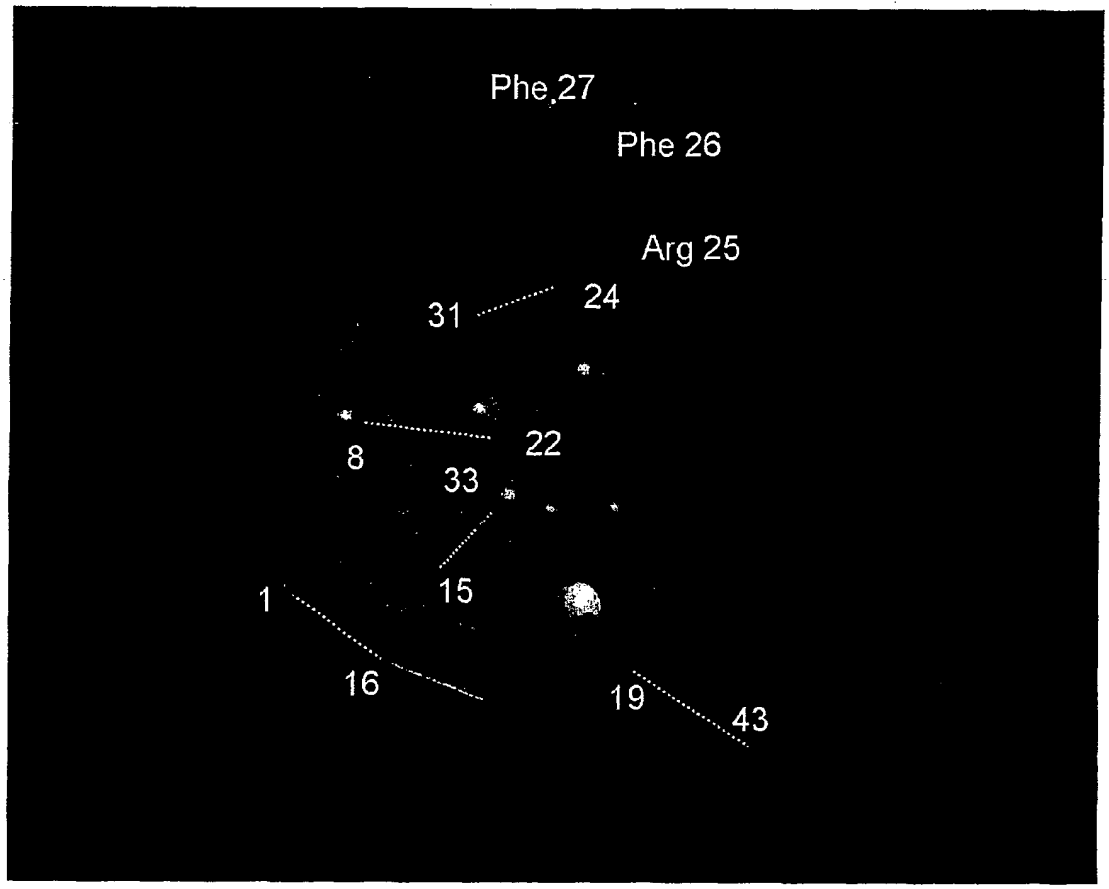

FIG._1B

FIG._2A

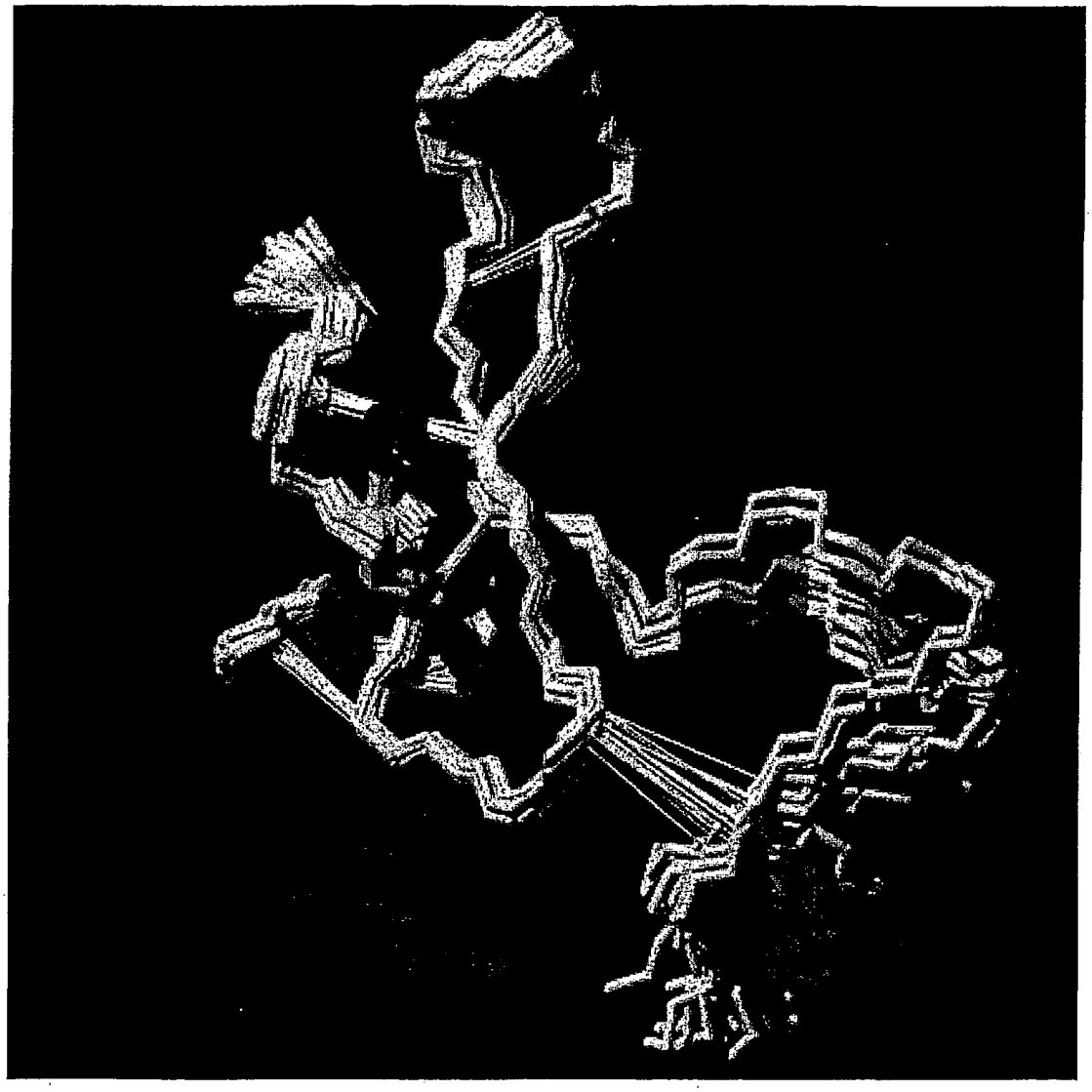

FIG._2B

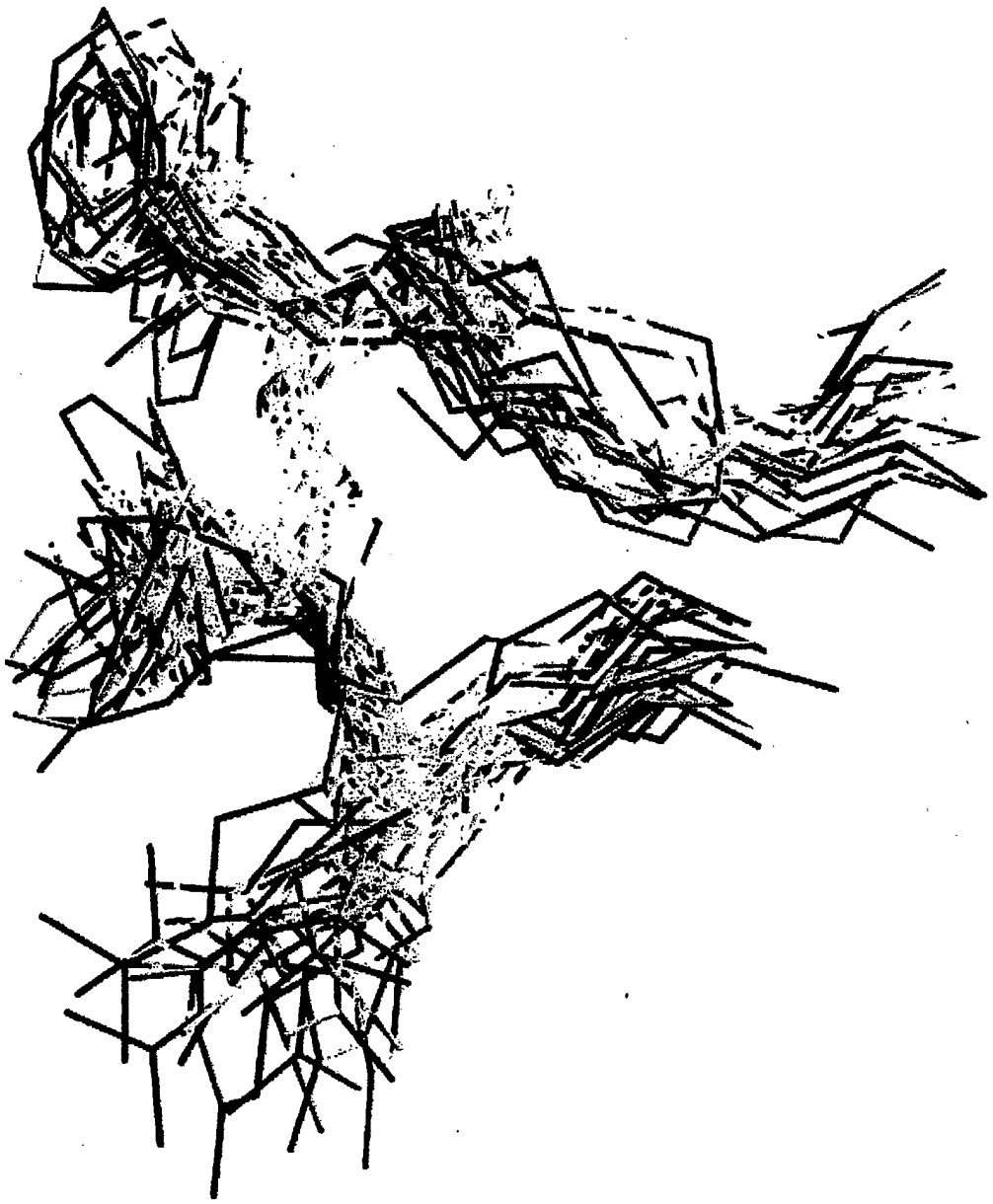

FIG._3

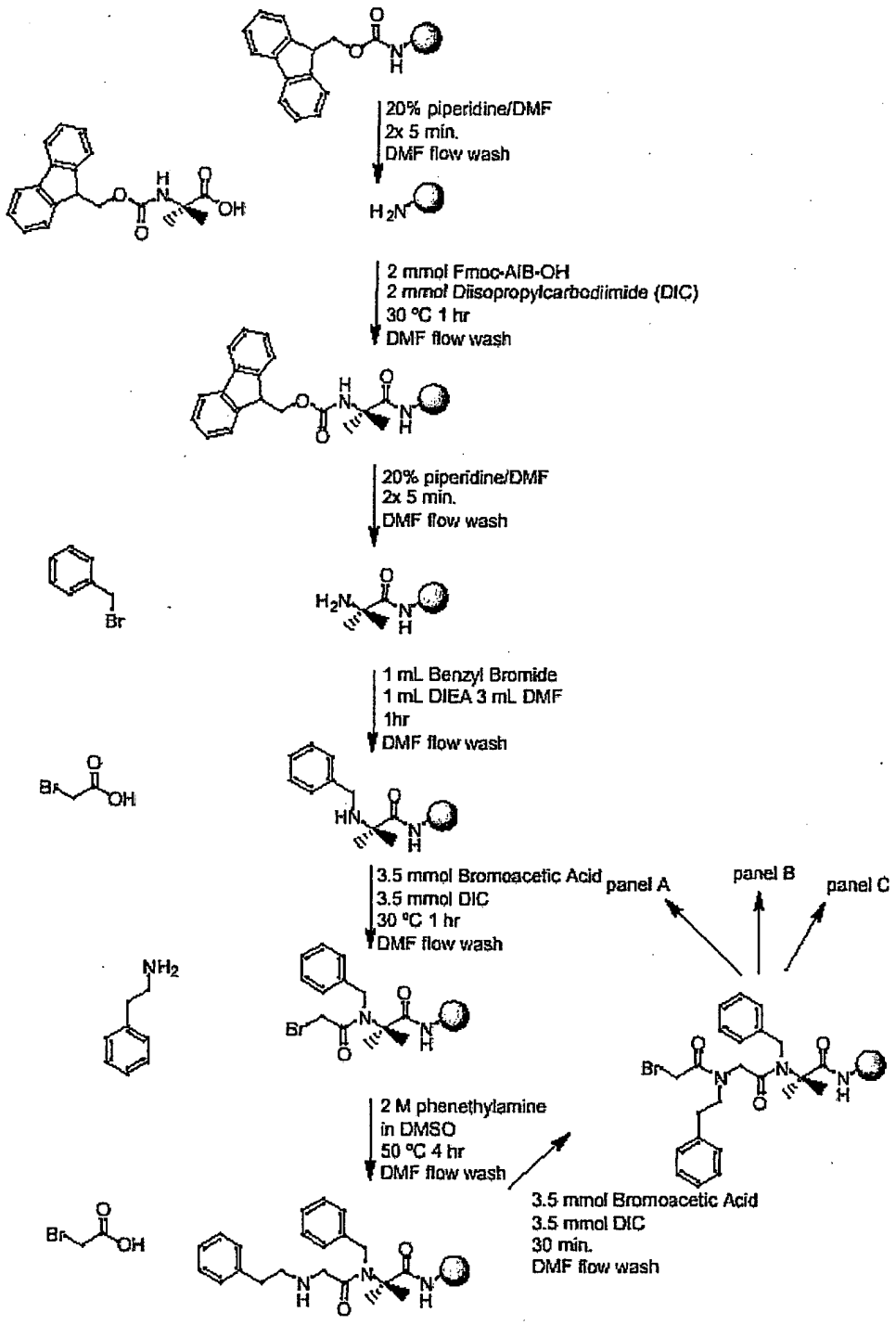

Figure 4 panel 1

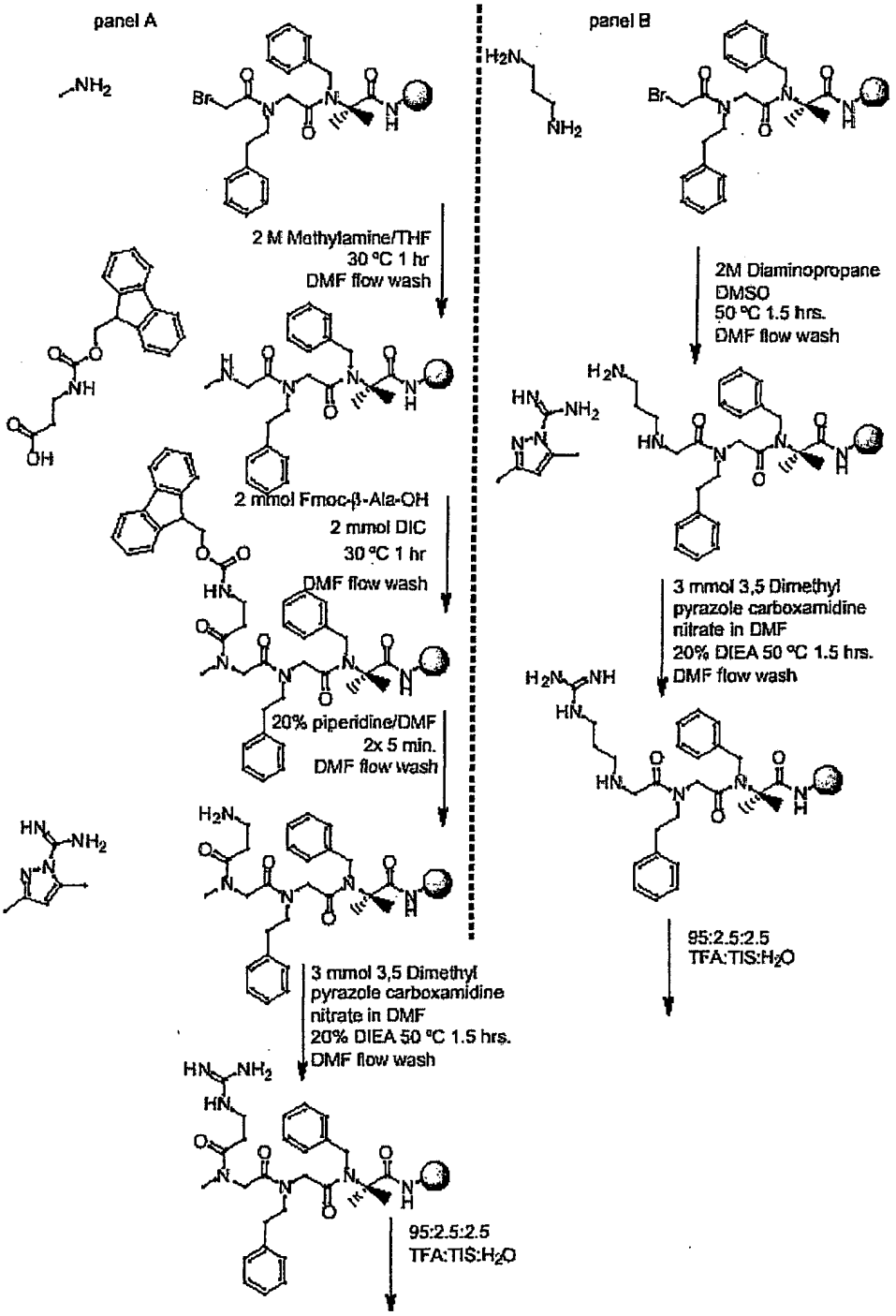

Figure 4 panels a and b

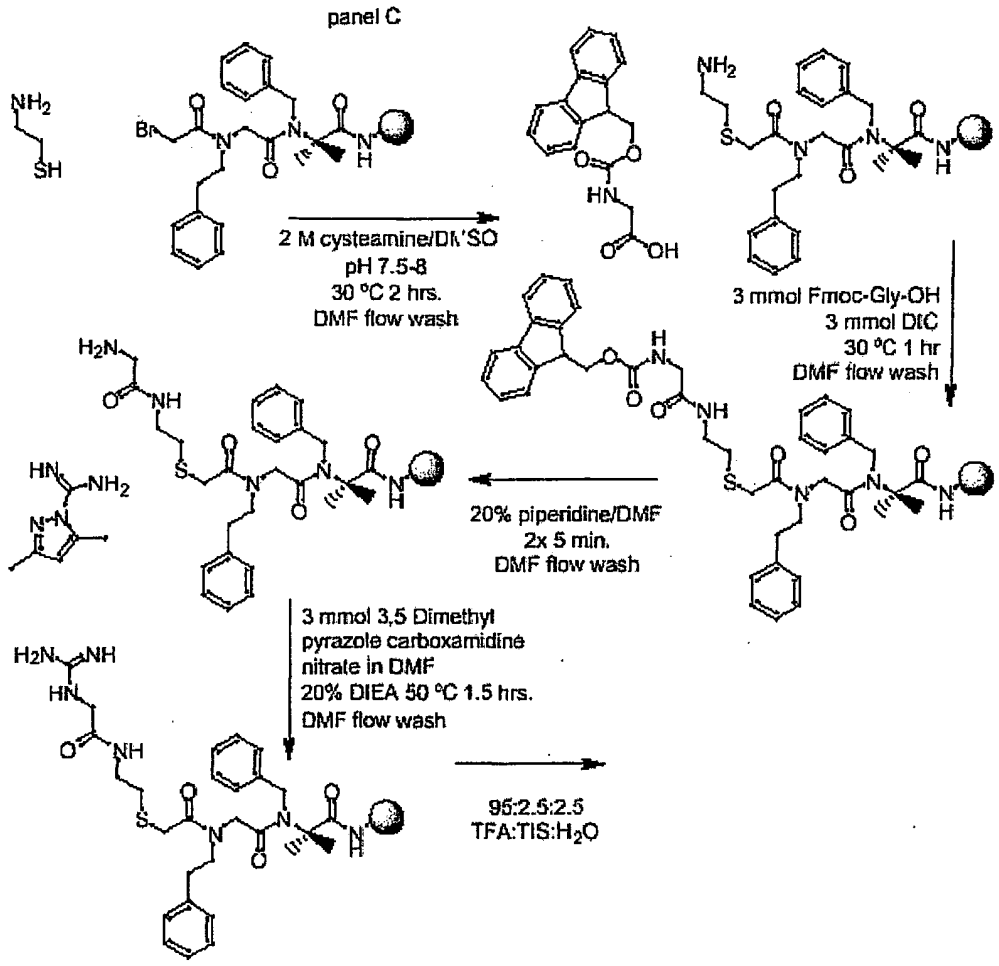

Figure 4 panel c

SEQUENCE LISTING

<110> The Regents of the University of California

<120> METHODS AND COMPOUNDS FOR MODULATING MELANOCORTIN RECEPTOR-LIGAND BINDING

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65                  70                  75                  80

Pro Arg Ser Ser Arg Arg Cys Val Arg Leu His Glu Ser Cys Leu Gly
                85                  90                  95

Gln Gln Val Pro Cys Cys Asp Pro Cys Ala Thr Cys Tyr Cys Arg Phe
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20 25 30

Cys

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WO 01/82953 A2

(54) Title: METHODS FOR TREATMENT OF DISEASES ASSOCIATED WITH INFLAMMATION UNDER NON-ISCHEMIC CONDITIONS

(57) Abstract: The present invention relates to a method for treatment or prevention of a non-ischemic condition in one or more organs, the method comprising administering an effective dosage of α-MSH and/or an α-MSH equivalent and/or EPO and/or an EPO equivalent to a person in need thereof. In particular, the invention relates to the treatment of inflammation under non-ischemic conditions. Non-limiting examples of such conditions are: Asthma, arthritis, psoriasis, infections, systemic lupus erythematosus, Systemic Sclerosis, allergic rhinitis, allergic and non-allergic conjunctivitis. Moreover, inflammatory diseases also include allergic and non-allergic dermatitis.

Methods for treatment of diseases associated with inflammation under non-ischemic conditions

FIELD OF INVENTION

The present invention relates to a method for treatment or prevention of inflammation in a non-ischemic condition in one or more organs, the method comprising administering an effective dosage of α-MSH and/or an α-MSH equivalent and/or EPO and/or an EPO equivalent to a person in need thereof. In particular, the invention relates to the treatment of inflammation under non-ischemic conditions.

The present invention relates to such treatment with respect to all organs in the human or animal body, and in particular to treatment of airways, lung, kidney and urinary tract. In this respect, 1) single compound treatment (either α-MSH or EPO) of e.g. asthma, ureteral obstruction and nephritic syndrome being non-ischemic conditions, and 2) combination treatment with α-MSH equivalents and epoetin-alpha of asthma, ureteral obstruction and nephritic syndrome are contemplated. However, it appears that treatment with a combination of α-MSH and/or an α-MSH equivalent and EPO and/or an EPO equivalent has a marked effect which is significantly better than single compound treatments. Accordingly the invention also relates to a pharmaceutical composition including a kit comprising the combination.

BACKGROUND

Melanocortins

Melanocortins are proopiomelanocortin-derived mammalian peptide hormones that include adrenocorticotropic hormone [ACTH (1-39)], α- melanocyte-stimulating hormone [α-MSH (1-13)], and related amino acid sequences including β- and γ-MSH. Melanocortin peptides have potent antiinflammatory/anticytokine activity (Lipton and Catania *Immunol.Today*, 18: 140-145, 1997). Melanocortins excert at least some of their effect via stimulation of melanocortin receptors. For melanocyte stimulating hormones (MSH) the action is in part ascribed to binding and activation of type 1-5 melanocortin receptors (MC1-MC5).

Melanocortins have a variety of functions including immunomodulation, anti-inflammation, body temperature regulation, pain perception, aldosterone synthesis, blood pressure regulation, heart rate, vascular tone, brain blood flow, nerve growth, placental development, synthesis/release of a variety of hormones such as aldosterone, thyroxin, prolactin, FSH. ACTH has a major effect on stimulating steroidoneogenesis. Also α-MSH induces pigment formation in skin.

Five genes encoding melanocortin receptor subtypes have been identified (MC-receptor type 1-5). The MC receptors belong to the class of G-protein coupled receptors and have seven membrane spanning domains. All receptor subtypes involve increased production of camp to excert their actions. Type 2 receptor (MC2) represent the ACTH receptor whereas the other subtypes are melanocyto stimulating hormone receptors (MSH-receptors).

A series of studies have been performed on the MC receptors in a variety of tissues. Type 1 receptor (MC1), to which α-MSH binds with great affinity, is known to be expressed in several tissues and cells such a brain, including astrocytes, testis, ovary, macrophages, neutrophils. However MC1 is likely to be expressed in an even wider range of tissues although this remains to be established. The selectivity for the MC=s to bind different melanocortin peptides vary. MC1 binds with great affinity α-MSH and with lower affinity also β-MSH, γ-MSH and ACTH. MC2 has been reported only to bind ACTH but non of the MSH peptides. The highest affinity for the ligands of the other receptors including γ-MSH (MC3-receptor), β-MSH (MC4-receptor). In contrast MC5 binds with much lower affinity the MSH peptides but with the same pattern as MC1 (i.e. highest affinity for α-MSH).

It is important to emphasize that a number of actions of MSH peptides, especially α-MSH, are not fully established with respect to which receptors are involved. The anti-inflammatory action of α-MSH has been speculated to involve a variety of processes including interference with NO production, endothelin-1 action, interleucin 10 formation, which again is linked to MC1 receptors expressed in macrophages, monocytes.

α-MSH has also been shown to be important in a variety of infllamtory processes (Lipton and Catania 1997): 1) Inhibit chemotactive migration of neutrophils (Catania1996). 2) α-MSH including analogs inhibit the release of cytokine (IL-1, TNF-α) in response to LPS

treatment (Goninard1996). 3) Inhibit TNF-α in response to bacterial endotoxin (Wong, K.Y. et al. *Neuroimmunomodulation*, 4: 37-41,1997). 4) ICV or IP administration of α-MSH inhibit central TNF-α production by locally administered LPS. 5) α-MSH has been shown to reduce the inflammation in experimental inflammatory bowel disease (Rajora, N. et al. *Peptides*, 18: 381-385, 1997), ishemia-induced acute renal failure(Star, R.A. et al. *Proc.Natl.Acad.Sci.U.S.A*, 92: 8016-8020, 1995). 6) α-MSH also have some protective effect by inhibiting the induction and elicitation of contact hypersensitivity and induces hapten tolerance, and it is speculated that α-MSH may mediate important negative regulation of cutaneous inflammation and hyper-proliferative skin diseases (Luger, T.A. *J.Investig.Dermatol.Symp.Proc.*, 2: 87-93, 1997. To this end α-MSH causes increased IL-8 release from dermal microvasculature endothelial cells (Hartmeyer, M. *J.Immunol.*, 159: 1930-1937, 1997).

Erythropoetin (EPO)

The cellular adaptation to hypoxia involves many changes in gene expression, such as those of erythropoietin (Epo), vascular endothelial growth factor (VEGF), glycolytic enzymes, and tyrosine hydroxylase. Several reports have demonstrated that both oxygen sensing and chemical signaling occur via a common pathway that leads to the activation of hypoxia-inducible factor-1 (HIF-1), a transcription factor which is induced over a physiologically relevant range of oxygen tensions Epo is a 34-kDa glycoprotein hormone which has been characterized as the principal regulator of erythropoiesis and was thought to be exclusively produced in fetal liver and adult kidney in response to hypoxia . The molecular biology of the oxygen sensing mechanism underlying the transcriptional activity of Epo has been intensively investigated in HepG2 and Hep3B human hepatoma cell lines. In addition to transcriptional activation by HIF-1, mRNA stabilization has been found to account for an accumulation of Epo mRNA. Agents such as cobalt chloride (CoCl2) and desferrioxamine (DFX) are able to mimic the hypoxia-induced Epo transcription.

Indirect evidence has been provided to indicate that redox-mediated processes are likely to be involved in the induction of the EPO gene. Thus, iron and reactive oxygen species might play a critical role in the oxygen sensing mechanisms involved in the regulation of the expression of the EPO gene. Recent reports suggest that, along with its role in erythropoiesis, EPO might be of biological significance in the central nervous system. In vivo, EPO mRNA is expressed in both rodent and primate brain tissues and its expression

is increased following hypoxia. Taken together, several findings imply that EPO acts on neurons in a paracrine way. This notion has been supported by the in vitro and in vivo europrotective effects of Epo. Several groups (Sadamoto, Y. et al. *Biochem. Biophys. Res. Commun.*, 253: 26-32, 1998, Sakanaka, M. et al. *Proc.Natl.Acad.Sci.U.S.A*, 95: 4635-4640, 1998, Bernaudin, M. et al. *J.Cereb.Blood Flow Metab*, 19: 643-651, 1999) have shown that the direct administration of EPO to the central nervous system of mice, rats, and gerbils to some extent reduces neuronal death and prevents learning disability associated with cerebral ischemia.

BRIEF DESCRIPTION OF THE INVENTION

The effect of treatment with i.v. α-MSH equivalents alone, epoetin alone or α-MSH equivalents and epoetin combined was established in models of lung inflammation, systemic inflammation, kidney inflammation, and diseases of the urinary tract including the bladder. Models include LPS administration, ureteral obstruction, and aminoglycoside induced nephrotic syndrome. Various functional parameters were determined and the expression levels of relevant transporters were monitored to establish the effect of these compounds in these settings.

Acute/subacute LPS-inhalation induced lung and airway inflammation.
Treatment with the compounds significantly prevented the neutrophil and eosinophil airway and lung infiltration. Subacute systemic administration of LPS gave multiple organ inflammation including renal inflammation and treatment with the compounds significantly prevented the development of severe renal failure. Functional parameters of kidney function and of renal transporter expression were determined as parameters of treatment efficiency. Temporal ureteral obstruction for 24 hours (uni or bilateral) followed by release of obstruction for various time periods (1-30 days) induced significant changes in kidney and urinary tract function. Markers include functional parameters, downregulation of renal water and sodium transporters. Treatment with i.v. α-MSH -equivalents alone, epoetin alone or α-MSH and epoetin combined markedly reduced the downregulation of the renal marker proteins and prevented the reduction in kidney and urinary tract function.

In rat models of purimycin or adriamycin induced nephrotic syndrome ascites and proteinuria developed as prominent signs of severe nephritic syndrome. Other markers

included downregulation of renal water channels (aquaporins) and sodium transporters. Treatment with i.v. α-MSH-equivalents alone, epoetin alone or α-MSH and epoetin combined markedly reduced the downregulation of the renal marker proteins and reduced proteinuria and ascites production.

Inflammation of airways and lung is often associated with swelling of airway and lung tissue and infiltration of airway and lung tissue with leucocytes including neutrophils, eosinophils or basophils and mast cells. This is well established and seen in extrodinary common conditions such as common cold, airway infection, and pulmonary infections, but also in conditions associated with allergy including allergic rhinitis, asthma or other allergic conditions. Moreover this is also seen in acute diseases associated with inflammation of lung and airways as well as in chronic obstructive pulmonary disease (COPD). At least three conditions contribute to COPD. (1) Chronic bronchitis is an inflammatory condition in which neutrophils, CD8+ T-lymphocytes and CD68+ monocytes/macrophages predominate. The condition is defined clinically by the presence of chronic cough and recurrent increase in bronchial secretion sufficient to to cause expectoration. There is enlargement of mucus-secreting glands and goble cell hyperplasia, which can occur in the absence of airflow limitation. (2) Adult chronic bronchiolitis is an inflammatory condition of small bronchi and bronchioli in which here are CD8+ and macrophages. (3) Emphysema is an inflammatory condition of the alveoli in which neutrophils, T-lymphocytes, and macrophages/monocytes are involved, associated with the release of excessive amounts of elastases from neutrophils.The course of COPD is characterized by intermittent exacerbations of the disease. In an axcerbation, there is also an significant influx of eosinophils into the tissue contributing to the inflammation. COPD is the fourth leading cause of death in the United States. The incidence, morbidity, and mortality of COPD is rising throught the world. The total economic cost of COPD in the US in 1993 was estimated to be over $US15.5 billion. Treatments effectively reducing or stopping the progress of COPD are needed. The ideal therapy in COPD would be compounds capable of reducing the influx of neutrophils into the airways or shrinking the enlarged mucous producing glands. As the inflammation continues natural repair processes start, resulting in peribronchial fibrosis, loss of lung elasticity and emphysema.

Also inflammation seen in other organs (either local) or attributed to systemic or more widespread inflammation is a feature associated with many diseases including infections, allergy, rheumatic diseases, cancer and other conditions and diseases or as a side-effect of drugs or poisoning.

In some cases of systemic inflammation this often involves inflammation or affection of the kidney leading to renal dysfunction or renal failure often seen in severe life-threatening conditions. The reduction in creatinine clearance or other renal functional parameters are indicative of such systemic inflammation.

Bilateral ureteral obstruction (BUO) is associated with reduction in renal functions. Characteristically, long-term loss of urinary concentration capacity is a common finding in both children and adults. The present inventors and others have shown that BUO and release of BUO in rats is associated with the onset of a dramatic postobstructive diuresis (POD) and a reduction in urinary concentrating capacity. Importantly, it was demonstrated that a BUO and release of BUO in rats was associated with a marked reduction in the protein expression of aquaporin-2 (AQP2), AQP3 and AQP1. Unilateral ureteral obstruction (UUO) is a model of renal injury characterized by progressive tubulointerstitial fibrosis and renal damage, while relatively sparing the glomerulus and not producing hypertension or abnormalities in lipid metabolism. Irrespective of the underlying cause, many kidney diseases lead to tubulointerstitial inflammation and eventual interstitial fibrosis with permanent loss of renal function. Most medical investigators agree that new therapeutic strategies should be targeted at developing effective methods for inhibiting renal fibrogenesis. The mechanisms responsible for UUO-induced kidney fibrosis are not well understood. However, prolonged obstruction induces progressive renal fibrosis with dysfunction, which cannot be readily restored even with removal of the obstruction. Under these circumstances, pharmacotherapeutic intervention needs to be developed to reverse or halt the progression of the renal dysfunction that occurs as a consequence of the obstruction. Furthermore, the development of progressive interstitial fibrosis represents a final common pathway associated with a variety of kidney disorders that can lead to functional insufficiency. Thus, the search for effective treatment preventing the progression is of great importance not only for elucidating the mechanism of UUO-induced fibrosis, but also for alleviating the renal fibrosis seen under various conditions with chronic renal failure (CRF).

UUO results in changes in renal hemodynamics, infiltration of the kidney by macrophages, and subsequent fibrosis of the tubulointerstitium. Many of the pathophysiological alterations associated with renal disease are driven by the intercrine, autocrine, paracrine, and endocrine effects of angiotensin II and it has been have demonstrated that angiotensin II production is rapidly stimulated following the onset of ureteral obstruction.

Angiotensin II, in turn, upregulates the expression of other factors including transforming growth factor- (TGF-), tumor necrosis factor- (TNF-α), nuclear factor-B (NF-B), adhesion molecules, and chemoattractants , matrix proteins , and -smooth muscle actin (-SMA). The role of TNF- in the pathophysiology of obstructive uropathy, when compared with angiotensin II, is not well understood. In rats, pharmacological manoeuvers has been applied to inhibit angiotensin II formation or its biological action through receptor inhibition. No such pharmacological treatments are available to decipher the biological actions of TNF-. Two different cell surface receptors exist for TNF-, which are designated TNFR1 and TNFR2, that are derived from separate gene products. Moreover it has been shown that TNF- contributes, in part, to changes in interstitial volume, myofibroblast differentiation, and NF-B activation in the kidney during ureteral obstruction and are mediated through both the TNFR1 and TNFR2 gene products (mouse study). Thus the angiotensin II and TNF- systems appear to interact with each system, contributing to overall renal fibrosis. Also apoptosis plays a role and it has been shown that the expression of apoptotic and chemokine genes are significantly upregulated in UUO, and bioflavonoids and angiotensin inhibitors are able to attenuate the expression of these genes and thus may be beneficial in renal protection.

There are no really good treatment of these conditions: 1) The effect of anti-inflammatory treatment with corticosteroids (methylprednisolone) in patient with urinary tract obstruction caused by stones showed that treatment alone did not affect stone passage but combined treatment with the calcium antagonist treatment (nifedipine + methyl prednisolone) facilitated ureteral stone passage. In a few other studies it has been shown that corticosteroid treatment most likely is able to reduce the oedema of the ureteral wall associated with ureteral obstruction. 2) The effect of nonsteroidal anti-inflammatory treatment with cyclooxygenase inhibitors such as indomethacin, toradol and sulindic acid, agents which block prostaglandin synthesis, all have been shown to have some beneficial effect on stone passage and reduce pains associated with ureteral obstruction. The mechanism involved are speculated to be due to a direct effect on ureteral contraction, reduced oedema of the ureteral wall which in turn may reduce ureteral pressure. The effect to reduce pain by effecting ureteral pressure may also be due to a direct side effect on renal function where GFR and RBF are reduced due to blockade of the effects of vasodilating prostaglandins on renal hemodynamics. Thus a drug with major effect on renal and urinary tract function during obstruction and other kidney disorders associated with fibrosis etc is warranted.

Another common cause of renal failure is nephrotic syndrome, which is caused by glomerular damage and can be a result of treatment with drugs such as adriamycin or purimycin (PAN) aminoglycosides (and other drugs) but the underlying cause of most cases remains unidentified and many patients progress into renal failure (for references see). Nephrotic syndrome is associated with severe proteinuria, systemic edema including ascites, hypoproteinemia and hyperlipidemia. It is also associated with decreased urinary concentrating capacity and dilutional ability, and severe sodium and water retention is a cardinal feature in nephrotic syndrome leading to ascites, and progression into renal failure. The intrarenal factors leading to the dysregulation of kidney function are not well understood and currently there is no good treatment that prevents the progression of kidney damage. The glomerular changes and possibly also the tubular changes that results in glomerular and tubular dysfunction leading to heavy proteinuria, sodium and water retention and massive peripheral edema have been speculated to be secondary to infiltration with neutrophils, macrophages and monocytes (and induction of many cytokines and adhesion molecules), although this remains less well defined. Also insufficient proliferation and apoptosis in glomerular epithelial cells may be involved in the progression. Thus an inflammatory response is likely to contribute during some stages of the development of renal failure.

A number of renal transporters and channels have been shown to be dysregulated in association with nephrotic syndrome and this is likely to contribute to the derangement in kidney function. It has been speculated that the decrease in transporter expression in experimental nephritic syndrome is a direct effect of the causing agents (e.g. adriamycin or PAN) on the renal tuble and glomerual epithelial cells. The mechanism by which this agent produces obliteration of the foot processes of glomerular podocytes is not known, but the effect presumably involves impairment of the vesicle trafficking processes involved in maintaining the complex shape of these cells. It appears possible that the decline in ion transporter and water channel expression induced by adriamycin is a consequence of a similar impairment of trafficking in renal tubule cells although this is not established.

Nephrotic syndrome differs markedly from ishemia-induced acute renal failure, which is due to reduced or complete arrest in blood supply to the kidney(s). Nephrotic syndrome is often caused (as described above) by drugs (aminoglycosides and other antibiotics), infections, autoimmune disease, connective tissue diseases, cancer and many immune-

mediated forms of glomerulonephritis and is never induced by ischemia. Ischemia is mainly associated with tubular damage whereas nephritic syndrome is mainly a glomerular disease with secondary affection of the tubular system. Thus the etiology is completely different.

As decribed above (and in the Lipton review) α-MSH is likely to exert its effect via MC-1 receptors expressed in inflammatory cells (including neutrophils) or directly in the epithelium (not known). The effects of epoitin outside its effect in stimulating erythropoesis is virtually undefined but may include leads activation of hypoxia-induc-ible factor-1 (HIF-1), a transcription factor which is induced over a physiologically relevant range of oxygen tensions. EPO receptors have been found in multiple tissues including kidney and it is speculated that EPO acts via this receptor although binding to other receptors cannot be excluded.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a method for treatment or prevention of an inflammatory condition in one or more organ(s) or tissue(s) such as treatment or prevention of the various conditions above. The organs include, but are not limited to, the airways and lung, the kidney and urinary tract system and the prostate. The method comprises administration of an effective dosage of α-MSH and/or of an α-MSH equivalent to the individual in need thereof. In another embodiment, the invention relates to the administration of an EPO or EPO equivalent to the individual in need thereof. In a still further and preferred embodiment, the invention relates to the use of both EPO and/or EPO equivalent and α-MSH and/or an α-MSH equivalent for the treatment or prevention of an inflammatory condition in one or more organ(s) or tissue(s) wherein a combination is administered to the individual in need thereof. In the present context, the term medicament may accordingly represent either α-MSH and/or α-MSH equivalent, EPO and/or EPO equivalent as well as any combination thereof. An example of a such combination is α-MSH together with an EPO equivalent.

By the term "an inflammatory condition" is in the present context meant a condition in which mechanisms such as reaction of specific T lymphocytes or antibody with antigen causes the recruitment of inflammatory cells and endogenous mediator chemicals. In some cases, the normal function of the organ or tissue will be altered by an increase in

vascular permeability and/or by contraction of visceral smooth muscle. Such inflammatory conditions may give rise to inflammatory diseases.

Inflammatory diseases include the following diseases (non-limiting list): Arthritis, including diseases associated with arthritis), osteoartritis, rheumatoid arthritis; spondylarthropathies (e.g. ankylosing spondilitis), reactive arthritis (including arthritis following rheumatic fever), Henoch-Schonlein purpura, and Reiter's disease. Moreover inflammatory diseases include connective tissue disorders such as systemic lupus erythematosus, polymyositis/dermatomyositis, systemic sclerosis, mixed connetive tissue disease, polymyalgia rheumatica, and other types of vasculitis, crystal deposition diseases (including gout), pyrophosphate arthropathy, acute calcific periarthritis. Moreover inflammatory diseases include infective arthritis, juvenile arthritis (Still's disease), psoriasis, osteoarthritis, osteoarthritis secondary to hypermobilty, congenital dysplasias, slipped femoral epiphysis, Perthes'disease, intra-articular fractures, meniscectomy, obesity, recurrent dislocation, repetitive actions, crystal depositions and diseases and *meatobilic abnormalities of cartilage including pyrophosphate arthropathy, ochronosis*, heamochromatosis, avascular necrosis including Sickle Cell disease, therapy with corticoids or other drugs, Caisson disease, septic or infectios arthitis (including tuberculous arthritis, meningococcal arthristis, gonococcal arthritis, salmonella arthritis), Lymes disease, infective endocarditis (including endocarditis induced by Stretococcus viridans, Enterococcus Faecalis, Staphylococcus aureus, Staphylocossus epidermidis, Histoplasma, Brucella, Candida and Aspergellus species and Coxiella Burnetii), viral arthritis (including infection with rubella, mumps, hepatitis B, HIV or Parvovirus), or recurrent heamarthrosis. Moreover inflammatory diseases include connective tissue diseases such as systemic lupus erythematosus, polymyositis/dermatomyositis, systemic sclerosis, mixed connetive tissue disease, sarcoidosis and primary Sjogrens syndrome including keratoconjunctivitis sicca. Moreover inflammatory diseases include vasculitis such as infective vasculitis due to infections with bacterial species including spirochaetal diseses as Lyme disease, syphilis, rickettsial and mycobacterial infections, fungal, viral or protozoal infections. Moreover inflammatory diseases include non-infective vascultitis including Takayasu's arteritis, Giant Cell Arteritis (Temporal arteritis and polymyalgia rheumatica), Buerger's disease, polyarteritis nodosa, microscopic polyarteritis, Wegener's granulomatose, Churg-Strauss syndrome, Sarcoidosis, vasculitis secondary to connective tissue diseases including Systemic Lupus Erythematosus, Polymyositis/Dermatomyositis, Systemic Sclerosis, Mixed Connetive Tissue Disease, sarcoidosis and Primary Sjogrens

syndrome. Moreover inflammatory diseases include vasculitis secondary to rheumatoid arthritis.

Moreover inflammatory diseases include non-infective vasculitis secondary to hypersensibility and leucocytoclastic vascultis including Serum Sickness, Henoch-Schonlein purpura, Drug induced vasculitis, essential mixed cryoglobulinaemia, hypocomplentaemia, Vascultis associated with other kinds of malignancy, inflammatory bowel disease and primary biliary cirrhosis, Goodpsture syndrome.

Moreover inflammatory diseases include all kinds of arthtitis in children such as Juvenile Chronic arthritis including Still's disease, juvenile rheumatoid arthritis, juvenile ankylosing spondylitis.

Moreover inflammatory diseases include all kinds of depostion diseases as Gout, pyrophosphate arthopathy and acute calcific periarthritis.

Moreover inflammatory diseases include all kind of inflammatory conditions causing backpain including infections, septic discitis, tuberculosis, malignancies (such as matastases, myeloma and others), spinal tumours, ancylosing spondylitis, acute disc prolapse, chronic disc diseaase/osteoarthritis, osteoporosis, and osteomalacia. It also includes Pagets disease, hyperparathyroidism, renal osteodystrophy, spondylolisthesis, spinal senosis congenital abnormalities and fibromyalgia.

Moreover inflammatory diseases include all kinds of soft-tissue rheumatism including bursitis, tenosynovitis or peritendonitis, enthesitis, nerve compression, periarthritis or capsulitis, muscle tension and muscle dysfunction.

Moreover inflammatory diseases include inflammatory diseases of the gastrointestinal system (including stomatitis of all kinds, pemfigus, bulloid pemphigoid and benig mucous membrane pemphigoid), salivary gland diseases (such as sarcoidosis, salivary duct obstruction and Sjogrens syndrome), inflammaton of the oesophagus (e.g. due to gastro-oesophagel reflux or infections with candida species, herpes simplex and cytomegalus virus), inflammatory dieseases of the stomach (including acute and chronic gastritis, helicobacteer pylori infection and Mentriers disease), inflammation of the the small intestine (including coeliac disease, gluten sensitive enteropathy, dermatitis herpitiformis,

tropical sprue, Whipple's diease, rdiation enteritis, systemic amyloidosis, connective tissue disorders including systemic lupus erythematosus, polymyositis/dermatomyositis, systemic sclerosis, mixed connetive tissue disease and sarcoidosis), eosinophilic gastroenteritis, intestinal lympangiectasia, inflammatory bowel disease (including Chrons disease and ulcertive colitis), diverticular disease of the colon, and irritable bowel syndrome

Moreover, inflammatory diseases include upper and lower airway diseases such as chronic obstructive pulmonary diseases (COPD), allergic and non-allergic asthma, allergic rhinitis, allergic and non-allergic conjunctivitis. Moreover, inflammatory diesases also include allergic and non-allergic dermatitis.

A preferred embodiment of the invention relates to treatment of inflammatory conditions or diseases under non-ischemic conditions, i.e. conditions wherein there is substantially normal blood supply to the organ or organs in question.

One preferred target organ is airways and lungs known to be site for inflammation in acute respiratory diseases and in chronic and subchronic airway and lung diseases. Another preferred target organ is the kidney, including tubules and glomeruli and the urinary tract system comprising ureteres, bladder, and urethra. However, also other cell types such as the prostate may be involved in an inflammatory condition, which thereby is the subject for treatment by the method according to the invention.

The cells to be treated may be one or more cell types selected from macrophages, the reticulo endothelial system monocytes, neutrophil granulocytes, eosinophil granulocytes, basophil granulocytes, T-cells, B-cells, mast cells, and dendritic cells.

In its broadest concept the invention relates to any condition wherein the normal function of the organs or tissues is altered including conditions associated with ischemia, acute and/or chronic inflammation, allergy, rheumatic diseases, infection including viral, fungal, bacterial infections, prions and other microbes and infectious agents known in the art. The injury may include acute and chronic injury. Chronic injury includes situations of repetitive injuries alternating with periods of complete or partial recovery of the organ(s) or tissue(s) function. The invention also relates to injury, which is associated with implantation of one or more organs or other devices for transplantation. The organ can be from the individual

him or herself, the animal itself or from other individuals or animals. This includes: organ transplants, bone transplants, soft tissue implants (silicone implants), metal and plastic implants, or other medical implantable devices. Individual represents humans as well as other mammals.

In a further embodiment, the condition to be treated may be caused by a cancer or a by premalignant disorder having an impact on the organ, e.g. on the respiratory system including lung, bronchiole, upper airways, and/or on the heart and/or on the kidney and/or on the gastrointestinal system, including acute leukemia, chronic myelocytic leukemia, chronic lymphocytic leukemia, Hodgkin's disease, lymphosarcoma, myeloma, metastasizing carcinoma of any origin.

Furthermore, the condition to be treated may be caused by any disease selected from diabetes mellitus, conditions with increased fasting levels of LDL-Cholesterol, conditions with combined increased fasting levels of LDL-Cholesterol and triglycerid, conditions with increased fasting levels of triglycerid, conditions with increased fasting levels of HDL-Cholesterol, retroperitoneal fibrosis, lupus erythematosus, polyarteritis nodosa, sclerodermia, polymyositis, dermatomyositis, rheumatoid arthritis, anaphylaxis, serum sickness, hemolytic anaemia, and allergic agranulocytosis.

Many infections may have an influence on the tissue and disturb the normal function resulting in decreased performance which may be improved by administration of an effective dose of α-MSH and/or an α-MSH equivalent and EPO and/or an EPO equivalent. Such infections include infections by protozoa, virus, bacteria and fungus and include conditions such as AIDS, bacterial septicemia, systemic fungal infections, Rickettsial diseases, toxic shock syndrome, infectious mononucleosis, chlamydia thrachomatis, chlamydia psittaci, cytomegalovirus infection, campylobacter, salmonella, influenza, poliomyelitis, toxoplasmosis, Lassa Fever, Yellow Fever, billharziose, colibacteria, enterococcus, preteus, klebsiella, pseudomonas, staphylococcus aureus, staphylococcus epidermidis, candida albicans, tuberculosis, mumps, infectious mononucleosis, hepatitis and Coxackie virus

In a still further aspect, the condition to be treated may be associated with a chemical trauma involving one or more toxic substances and/or drugs. Such drugs include tricyclic

antidepressants, lithium salts, prenylamine, phenothizine derivatives, chemopreventive drugs including adriamycin. Also physical traumas including electromagnetic radiation may cause damages which can be alleviated by administration of an effective dose of an α-MSH and/or of an α-MSH equivalent and/or administration of an EPO and/or an EPO equivalent according to the present invention.

The condition, which may be treated according to the present invention may further include connective tissue disease such as scleroderma, systemic lupus erythematosus or by neuromyopathic disorders such as progressive muscular dystrophy of Duchenne's type, Friedreich's ataxia, and myotonic dystrophy. The condition may e.g. be related to the tissue of the intestine of the mammal.

The medicament in question may be administered therapeutically for treating an existing condition or prophylactically for preventing a progress of the condition, or of any symptom of the condition. It may be administered prophylactically for preventing the establishment of the condition or of any symptom of the condition. The α-MSH and/or α-MSH equivalent and/or rh-EPO and/or a rh-EPO equivalent may be administered as a single dosage, as continued administration including a regimen where specific dosages are prescribed for a shorter or longer duration, or as a sequential administration similarly with many treatment schedules for cancer therapy.

The origin of the condition may include anatomic abnormality of the tissue or organ, inflammatory diseases, and/or conditions caused by a chemical trauma including drugs such as adriamycin and other chemotherapeutics; electromagnetic radiation; renal and/or ureteric calculi, especially when the calculi occur frequently.

The preferred target organ according to the invention is airways, lung, ureteres, kidney, bladder, urethra and the prostate gland and wherein the tissue(s) is selected from the group of lymphoid tissues, mucosa, epithelium, and endothelium. However also inflammation in other organ systems or parts of organs may be succesfully treated by the methods according to the invention. Examples of such organs or organ systems which may be treated according to a method of the invention are skin, skeleton, brain and central nervous system, muscles, vessels, upper and lower respiratory tract, lungs and pleura, exocrine and endocrine glands, heart and pericardium, intestinal tract including

excretory ducts of liver and pancreas, liver, pancreas, genital organs and uterus, kidney and urinary tract including prostate.

An interesting embodiment relates to the treatment and/or prevention of infections in target organs including lung and airways but also cystitis, especially interstitiel cystitis, and cystitis limited to the mucosa. The infections and inflammation may be a microbial or non-microbial. The infection or inflammation may be local or in distinct organ regions or systemic.

Many diseases such as rheumatic diseases, retroperitoneal fibrosis, lupus erythematosus, polyarteritis nodosa, sclerodermia, polymyositis, dermatomyositis, rheumatoid arthritis, anaphylaxis, serum sickness, hemolytic anaemia, allergic agranulocytosis, may succesfully be treated according to the present invention.

Another important embodiment is the conditions wherein diabetes mellitus is involved.

Yet another important embodiment is conditions associated with inflammation of skin.

Examples of conditions to be treated by the methods according to the present invention is wherein the condition is lung and/or airways, kidney (recognized as renal failure, nephrotic syndrome) or complete or partial urinary tract obstruction, postoperative polyuria.

In one important embodiment the condition to be treated by the methods of the invention is associated with inflammation of lung and airways with infiltration of one or more of leucocytes including neutrophils, eosinophils, lymphocytes and monocytes but also macrophages, mast cells or basophils. These conditions include chronic obstructive pulmonary diseases with or without acute infections or worsening of inflammation, allergic diseases, astma. This also includes inflammation in the airways including upper airways caused by allergy or infection or other diseases.

In one embodiment the condition is associated with reduced renal function indicated by one or more of the following conditions; reduced renal blood flow, reduced glomerular filtration rate, reduced urinary concentrating ability, reduced urinary concentration capacity, reduced or increased urinary electrolyte excretion (such as sodium, potassium,

bicarbonate), reduced creatinine clearance which may be treated or prevented by the methods according to the invention.

Accordingly, the condition may be associated with dysregulation of one or more renal sodium transporters. Such downregulation of one or more renal sodium transporters may be of at least 50 %, such as at least 75%, compared to non-treatment. The sodium transporters may be selected from the group consisting of Na,K-ATPase, NHE-3, NaPi-2, BSC-1, TSC and ENaC's.

Also dysregulation of one or more renal aquaporins may characterize a condition to be treated according to the invention including downregulation of one or more renal aquaporins including aquaporins selected from aquaporins 1 to 12, preferably aquaporins 1 to 4.

The administration according to the use and method of the present invention may be any administration known in the art as may easily be recognised by the skilled person according to the individual situation. Accordingly, the administration may be selected from systemic administration; injection into tissue or into a to body cavity including joints; implantation into tissue or into a body cavity; topical application to the skin or to any gastrointestinal surface, or to a mucosal surface including the lining of body cavities.The administration may be selected from parenteral adminstration, including intraperitonal administration, intrathecal administration systemic administration, local administration, topical administration, transmucosal administration, transdermal administration and oral administration.

The α-MSH equivalent according to the present invention is preferably a substance acting on an α-MSH receptor and/or on a melanocortin receptor such as subtypes 1 to 5 (MC-receptors 1-5). Such substances are disclosed in e.g. EP 972522, WO 87/04623, WO 88/00833, WO 99/57148, WO 99/21571, WO 96/41815, US 5028592, US 5,731,408, US 5,830,994 and the references cited therein.

In a further important aspect, the α-MSH equivalent is a polypeptide having at least 3 amino acids including the following sequence Lys-Pro-Val, such as Gly-Lys-Pro-Val, or the following sequence His-Phe-Arg, and being able to act on an α-MSH receptor.

The treatment or prevention as described above is in a preferred embodiment performed with EPO and/or an EPO equivalent i.e. a substance acting on the EPO receptor. In this respect, the effective dosage of the unit of EPO and/or an EPO equivalent is lower than the dosage in which EPO is generally used for its known indications. The necessary dosage of EPO will generally be a completely non-toxic dosage for the individual.

By use of the combination of an α-MSH or α-MSH equivalent with rh-EPO and/or an EPO equivalent, a synergistic effect may be obtained. The synergism may during a treatment period be at least 5% or even higher such as at least 10%, preferebly at least 15% as measured according to the selected test system in an organ. It is believed that a synergistic effect of at least 20%, such as at least 25%, may be demonstrated by a treatment according to the invention. The test system may be any of the experimental protocols described herein.

Pharmaceutical formulations and compositions:

In the following examples of suitable compositions containing α-MSH and/or an α-MSH equivalent and EPO and/or EPO equivalent are given. Depending on the use of the α-MSH and/or an α-MSH equivalent and EPO and/or EPO equivalent, a composition may be a pharmaceutical or a cosmetic composition. In the following the term "pharmaceutical composition" is also intended to embrace cosmetic compositions as well as compositions belonging to the so-called grey area between pharmaceuticals and cosmetics, namely cosmeceuticals.

For the administration to an individual (an animal or a human) the substance(s) are preferably formulated into a pharmaceutical composition containing the substance(s) and, optionally, one or more pharmaceutically acceptable excipients.

The compositions may be in form of, e.g., solid, semi-solid or fluid compositions such as, e.g., but not limited to

bioabsorbable patches, drenches, dressings, hydrogel dressings, hydrocolloid dressings, films, foams, sheets, bandages, plasters, delivery devices, implants,

powders, granules, granulates, capsules, agarose or chitosan beads, tablets, pills, pellets, microcapsules, microspheres, nanoparticles, sprays, aerosols, inhalation devices,

gels, hydrogels, pastes, ointments, creams, soaps, suppositories, vagitories, tooth paste,solutions, dispersions, suspensions, emulsions, mixtures, lotions, mouthwash, shampoos, enemas,

kits containing e.g. two separate containers, wherein the first one of the containers contains the α-MSH and/or α-MSH equivalent and/or EPO and/or EPO equivalent and/or pharmaceutically acceptable excipients and the second container containing a suitable medium intended to be added to the first container before use in order to obtain a ready-to-use composition;and in other suitable forms such as, e.g., implants or coating of implants or in a form suitable for use in connection with implantation or transplantation.

The compositions may be formulated according to conventional pharmaceutical practice, see, e.g., "Remington: The science and practice of pharmacy" 20th ed. Mack Publishing, Easton PA, 2000 ISBN 0-912734-04-3 and "Encyclopedia of Pharmaceutical Technology", edited by Swarbrick, J. & J. C. Boylan, Marcel Dekker, Inc., New York, 1988 ISBN 0-8247-2800-9.

A pharmaceutical composition comprising an active substance serves as a drug delivery system. In the present context the term "drug delivery system" denotes a pharmaceutical composition (a pharmaceutical formulation or a dosage form) which upon administration presents the active substance to the body of a human or an animal. Thus, the term "drug delivery system" embraces plain pharmaceutical compositions such as, e.g., creams, ointments, liquids, powders, tablets, etc. as well as more sophisticated formulations such as sprays, plasters, bandages, dressings, devices, etc.

Apart from α-MSH and/or an α-MSH equivalent and/or EPO and/or EPO equivalent, a pharmaceutical composition for use according to the invention may comprise pharmaceutically or cosmetically acceptable excipients.

The choice of pharmaceutically acceptable excipients in a composition for use according to the invention and the optimum concentration thereof cannot generally be predicted and

must be determined on the basis of an experimental determination thereof. Also whether a pharmaceutically acceptable excipient is suitable for use in a pharmaceutical composition is generally dependent on which kind of dosage form is chosen. However, a person skilled in the art of pharmaceutical formulation can find guidance in e.g., "Remington: The science and practice of pharmacy" 20th ed. Mack Publishing, Easton PA, 2000 ISBN 0-912734-04-3.

A pharmaceutically acceptable excipient is a substance, which is substantially harmless to the individual to which the composition will be administered. Such an excipient normally fulfils the requirements given by the national drug agencies. Official pharmacopeias such as the British Pharmacopeia, the United States of America Pharmacopeia and the European Pharmacopeia set standards for well-known pharmaceutically acceptable excipients.

In the following is given a review on relevant pharmaceutical compositions for use according to the invention. The review is based on the particular route of administration. However, it is appreciated that in those cases where a pharmaceutically acceptable excipient may be employed in different dosage forms or compositions, the application of a particular pharmaceutically acceptable excipient is not limited to a particular dosage form or of a particular function of the excipient.

Parenteral compositions:

For systemic application, the compositions according to the invention may contain conventionally non-toxic pharmaceutically acceptable carriers and excipients according to the including microspheres and liposomes.

The compositions for use according to the invention include all kinds of solid, semisolid and fluid compositions. Compositions of particular relevance are e.g. solutions, suspensions, emulsions, gels, implantation tablets and implants.

The pharmaceutically acceptable excipients may include solvents, buffering agents, preservatives, humectants, chelating agents, antioxidants, stabilizers, emulsifying agents, suspending agents, gel-forming agents, diluents, disintegratig agents, binding agents, lubricants and wetting agents. For examples of the different agents see below.

Topical, trans-mucosal and trans-dermal compositions:

For application to the mucosa or the skin, the compositions for use according to the invention may contain conventionally non-toxic pharmaceutically acceptable carriers and excipients including microspheres and liposomes.

The compositions for use according to the invention include all kinds of solid, semi-solid and fluid compositions. Compositions of particular relevance are e.g. pastes, ointments, hydrophilic ointments, creams, gels, hydrogels, solutions, emulsions, suspensions, lotions, liniments, resoriblets, suppositories, enema, pessaries, moulded pessaries, vaginal capsules, vaginal tablets, shampoos, jellies, soaps, sticks, sprays, powders, films, foams, pads, sponges (e.g. collagen sponges), pads, dressings (such as, e.g., absorbent wound dressings), drenches, bandages, plasters and transdermal delivery systems.

The pharmaceutically acceptable excipients may include solvents, buffering agents, preservatives, humectants, chelating agents, antioxidants, stabilizers, emulsifying agents, suspending agents, gel-forming agents, ointment bases, suppositoriy bases, penetration enhancers, perfumes, skin protective agents, diluents, disintegratig agents, binding agents, lubricants and wetting agents. For examples of the different agents see below.

Oral compositions:

For application to the mucosa or the skin, the compositions for use according to the invention may contain conventionally non-toxic pharmaceutically acceptable carriers and excipients including microspheres and liposomes.

The composition for use according to the invention include all kinds of solid, semi-solid and fluid compositions. Compositions of particular relevance are e.g. solutions, suspensions, emulsions, uncoated tablets, modified-release tablets, gastro-resistant tablets, orodispersible tablets, efferverscent tablets, chewable tablets, soft capsules, hard capsules, modified.release capsules, gastro-resistant capsules, uncoated granules, effervescent granules, granules for the preparation of liquids for oral use, coated granules, gastro-resistant granules, modified-release granules, powders for oral administration and powders for the preparation of liquids for oral use.

The pharmaceutically acceptable excipients may include solvents, buffering agents, preservatives, humectants, chelating agents, antioxidants, stabilizers, emulsifying agents, suspending agents, gel-forming agents, diluents, disintegratig agents, binding agents, lubricants, coating agents and wetting agents. For examples of the different agents see below.

Examples of various agents:

Examples of solvents are but not limited to water, alcohols, vegetable or marine oils (e.g. edible oils like almond oil, castor oil, cacao butter, coconut oil, corn oil, cottonseed oil, linseed oil, olive oil, palm oil, peanut oil, poppyseed oil, rapeseed oil, sesame oil, soybean oil, sunflower oil, and teaseed oil), mineral oils, fatty oils, liquid paraffin, polyethylene glycols, propylene glycols, glycerol, liquid polyalkylsiloxanes, and mixtures thereof.

Examples of buffering agents are but not limited to citric acid, acetic acid, tartaric acid, lactic acid, hydrogenphosphoric acid, diethylamine etc.

Examples of preservatives for use in compositions are but not limited to parabens, such as methyl, ethyl, propyl p-hydroxybenzoate, butylparaben, isobutylparaben, isopropylparaben, potassium sorbate, sorbic acid, benzoic acid, methyl benzoate, phenoxyethanol, bronopol, bronidox, MDM hydantoin, iodopropynyl butylcarbamate, EDTA, benzalconium chloride, and benzylalcohol, or mixtures of preservatives.

Examples of humectants are but not limited to glycerin, propylene glycol, sorbitol, lactic acid, urea, and mixtures thereof.

Examples of chelating agents are but not limited to sodium EDTA and citric acid.

Examples of antioxidants are but not limited to butylated hydroxy anisole (BHA), ascorbic acid and derivatives thereof, tocopherol and derivatives thereof, cysteine, and mixtures thereof.

Examples of emulsifying agents are but not limited to naturally occurring gums, e.g. gum acacia or gum tragacanth; naturally occurring phosphatides, e.g. soybean lecithin;

sorbitan monooleate derivatives; wool fats; wool alcohols; sorbitan esters; monoglycerides; fatty alcohols;, fatty acid esters (e.g. triglycerides of fatty acids); and mixtures thereof.

Examples of suspending agents are but not limited to celluloses and cellulose derivatives such as, e.g., carboxymethyl cellulose, hydroxyethylcellulose, hydroxypropylcellulose, hydroxypropylmethylcellulose, carraghenan, acacia gum, arabic gum, tragacanth, and mixtures thereof.

Examples of gel bases and viscosity-increasing are but not limited to liquid paraffin, polyethylene, fatty oils, colloidal silica or aluminium, zinc soaps, glycerol, propylene glycol, tragacanth, carboxyvinyl polymers, magnesium-aluminium silicates, Carbopol®, hydrophilic polymers such as, e.g. starch or cellulose derivatives such as, e.g., carboxymethylcellulose, hydroxyethylcellulose and other cellulose derivatives, water-swellable hydrocolloids, carragenans, hyaluronates (e.g. hyaluronate gel optionally containing sodium chloride), and alginates including propylene glycol aginate.

Examples of ointment bases are but not limited to beeswax, paraffin, cetanol, cetyl palmitate, vegetable oils, sorbitan esters of fatty acids (Span), polyethylene glycols, and condensation products between sorbitan esters of fatty acids and ethylene oxide, e.g. polyoxyethylene sorbitan monooleate (Tween).

Examples of hydrophobic ointment bases are but not limited to paraffins, vegetable oils, animal fats, synthetic glycerides, waxes, lanolin, and liquid polyalkylsiloxanes.

Examples of hydrophilic ointment bases are but not limited to solid macrogols (polyethylene glycols).

Examples of powder components are but not limited to alginate, collagen, lactose, powder which is able to form a gel when applied to a wound (absorbs liquid/wound exudate).

Examples of diluents and disintegrating agents are but not limited to lactose, saccharose, emdex, calcium phosphates, calcium carbonate, calcium sulphate, mannitol, starches and microcrystaline cellulose.

Examples of binding agents are but not limited to saccharose, sorbitol, gum acacia, sodium alginate, gelatine, starches, cellulose, sodium coboxymethylcellulose, methylcellulose, hydroxypropylcellulose, polyvinylpyrrolidone and polyetyleneglycol.

Examples of wetting agents are but not limited to sodium laurylsulphate and polysorbate 80.

Examples of lubricants are but not limited to talcum, magnesium stearate, calcium stearate, silicium oxide, precirol and polyethylenglycol.

Examples of coating agents are but not limited to hydroxypropylcellulose, hydroxypropylmethylcellulose, polyvinylpropylidone, ethylcellulose and polymethylacrylates.

Examples of suppository bases are but not limited to oleum cacao, adeps solidus and polyethylenglycols.

The α-MSH and/or α-MSH equivalent and/or EPO and/or EPO equivalent is present in the medicament in an amount of 0.001-99%, typically 0.01-75%, more typically 0.1-20%, especially 1-10% by weight of the medicament.

By EPO equivalent according to the present invention is meant any substance, which has a functional effect on an EPO receptor.

The EPO and/or EPO equivalent may be present in the medicament in an amount of 0.001-99%, typically 0.01-75%, more typically 0.1-20%, especially 1-10% by weight of the medicament. The EPO and/or EPO equivalent is generally used in dosages, which are completely non-toxic to a human. By use of a combination of an EPO and/or EPO equivalent together with a unit dosage of α-MSH and/or an α-MSH equivalent, an effect of the combination may be obtained which is higher than the effect obtained with any of the substances administered alone.

With respect to the combination, it is possible to obtain an synergistic effect where the EPO and/or EPO equivalent and the α-MSH and/or α-MSH equivalent is administered independently of each other. The time span from the release of one of the active

ingredients to the organ or tissue in question until the other active ingredient is subjected to the tissue or organ may be several days, even 5 days or one week. However, the drugs are preferable administered within about 48 hours, preferably within 24 hours, such as within 12 hours. However, for practical reasons the active ingredients will normally be substantially co-administered with considerations to differences in pharmacokinetic properties and speed-of-action of the compounds.

In a further embodiment the present invention relates to a pharmaceutical composition comprising a unit dosage of EPO and/or EPO equivalent and a unit dosage of α-MSH and/or of an α-MSH equivalent, optionally together with a suitable pharmaceutical carrier. The carrier may be selected according to the specific use as disclosed above. In a further aspect, the composition may be specifically adapted for any of the uses and methods disclosed herein.

It is contemplated that the dose of α-MSH and/or α-MSH equivalent will be in the range of 1 ng to 100 mg pr. kg body weight, typically 1μg to 10 mg pr. kg body weight, more typically 10μg to 1 mg pr. kg body weight, such as 50-500 μg pr. kg body weight; and that the dose of EPO and/or EPO equivalent will be in the range of 0,001-10000 IU pr. kg body weight, typically 0,1-5000 IU pr. kg body weight, more typically 1-1000 IU pr. kg body weight, such as 50-500 IU pr. kg body weight.

A further aspect of the invention is a pharmaceutical kit comprising a unit dosage of EPO and/or EPO equivalent and a unit dosage of α-MSH and/or of an α-MSH equivalent optionally together with a suitable pharmaceutical carrier and optionally a description of the specific use. The kit may comprise the α-MSH equivalent or α-MSH in any of the forms described herein and the EPO and/or EPO equivalent may be in an identical for or in any other form. Accordingly, the α-MSH may be present in a device for sustained effect whereas the EPO may be present in the kit in a form suitable for injection. The specific kit may accordingly be designed for the individual treatment of prophylactic use.

LEGEND TO FIGURES

Fig 1. Panel A shows an immunoblot from whole kidney reacted with affinity-purified anti-aquaporin-1 (anti-AQP1) which revealed 29 kDa and 35-50 kDa AQP1 bands, representing non-glycosylated and glycosylated forms of AQP1. Panel B shows

densitometric analysis. In response to 24 hours of BUO and 5 hours release densitometric analysis of all samples from nontreated, α-MSH treated rats with 24-hour BUO and sham-operated controls revealed that AQP1 expression decreased from 100±22% in sham operated controls to 7±2% in BUO rats without α-MSH treatment,* P<0.05. α-MSH treatment significantly increased the level of AQP1 expression to 81±21% compared with nontreated rats with BUO, # P<0.05. Thus α-MSH treatment prevented the dramatic decrease in AQP1 expression in response to 24 hours of BUO and 5 hours of release.

Fig 2. Panel A: Glomerular filtration rate (GFR) did not differ among the three groups at baseline level. α-MSH treatment completely prevented the reduction in GFR (BUO+MSH: 705 ± 85 µl/min/100g vs. SHAM: 840 ± 105 µl/min/100g) 48 hours after release of BUO. Panel B: Effective renal plasma flow (ERPF) did not differ among the three groups at baseline level. α-MSH treatment completely prevented the reduction in ERPF (BUO+α-MSH: 2598 ± 129 µl/min/100g vs. SHAM: 2633 ± 457 µl/min/100g) 48 hours after release of BUO.

Fig 3. Determination of proteinuria levels in α-MSH or untreated PAN rats compared to SHAM rats at day 5 after PAN injection. A marked reduction is seen in proteinuria demonstrating a dramatic effect of α-MSH in preventing to a marked extent the severity in the developement of nephrotic syndrome

EXAMPLES

Experimental animals

Studies were performed on adult male Munich Wistar rats or Spraque Dawley rats (Møllegard Breeding centre Ltd., Eiby, Denmark). The rats were maintained on a standard rodent diet (Altromin, Lage, Germany) with free access to water. During the entire experiment, rats were kept in individual metabolic cages, with a 12:12h artificial light/dark cycle, a temperature of 21°C, plus/minus 2°C. Rats were allowed to acclimatize to the cages for 3 days prior to surgery.

Induction of acute lung inflammation

Studies were performed on male Spraque Dawley rats (Møllegard Breeding centre Ltd., Eiby, Denmark). The rats were maintained on a standard rodent diet (Altromin, Lage,

Germany) with free access to water. Inflammation of the lungs (asthma) was induced by LPS inhalation with the animals anesthetized using Midazolam and Hypnorm The LPS was administrated by connecting the rat to a semiclosed breathing system consisting of a MAXIN bottle filled with a mixture of LPS in saline connected to a standard inhalation chamber. Each animals is exposed for 20 min with 5 mg LPS. Twenty-four hours after exposure to LPS rats were killed. Subsequently, lavage was performed by washing the lungs with PBS (5 ml) 6 times. The lung tissue was then centrifuged for 10 min (1000 rpm) and resuspended in 0.5 ml. From each sample 2 blood smears were made. They were air-dryed, fixed and counterstained for differential cell counting.

Induction of acute systemic inflammation

Barrier-bred and specific pathogen-free female Wistar rats (210-230 g) were obtained from the Department of Experimental Medicine, Panum Institute, University of Copenhagen, Denmark. The animals were housed in a temperature (22-24° C) and moisture (40-70%) controlled room with a 12-hour light-dark cycle (light on from 6:00 A.M. to 6:00 P.M.). All animals were given free access to tap water and a pelleted rat diet containing approximately 140 mmol/kg of sodium, 275 mmol/kg potassium and 23 % protein (Altromin catalogue no. 1310, Altromin International, Lage, Germany). Rats were anesthetized with halothane-nitrous oxide and permanent medical grade Tygon catheters were implanted into the abdominal aorta and into the inferior caval vein via a femoral artery and vein. After instrumentation, the animals were housed individually. All surgical procedures were performed during aseptic conditions. To relieve postoperative pain, rats were treated with buprenorfin, 0.2 mg/kg body weight i.p. Two weeks later the rats were anesthetized with halothane-nitrous oxide and a osmotic minipump (Alzet 1003D)filled with a LPS solution was implanted into the abdomen. The mean infusion rate of LPS was 200 µg/kg/hour. All rats except the control rats received LPS infusion. N=5 in all groups.

Induction of bilateral ureteral obstruction

Adult Munich-Wistar rats were anesthetized with halothane and placed on a heating board under an operating microscope, Through a midline abdominal incision both ureters were exposed and occluded by placing a 5-mm piece of bisected polyethylene tubing (PE-50) around the midportion of each ureter. The ureter was then occluded by tightening the tubing with a 5-0 silk ligature. 24 hours later, the obstructed ureters were decompressed by removal of the ligature and the PE-50 tubing. Done in this manner, both ureters could

be completely occluded for 24 h and without evidence of functional impairment of ureteral function and rats followed up to several days after release of obstruction.

Induction of unilateral ureteral obstruction

Adult Munich-Wistar rats were anaesthetized with intraperitoneal sodium pentobarbital (50 mg/kg) and placed on a heating board under an operating microscope. Through a midline abdominal incision the left ureter was exposed and a 5 mm piece of bisected polyethylene tubing (PE-50) was placed around the midportion of the ureter. The ureter was then occluded by tightening the tubing with a 5-0 Silk ligature. Twenty-four hours later, the obstructed ureters were decompressed by removal of the ligature and the PE tubing. Using this technique the ureter could be completely occluded for 24 hours without evidence of subsequent functional impairment of ureteral function.

Induction of purimycin and adriamycin-induced nephrotic syndrome in rats

Nephrotic syndrome was induced by a single i.p. or s.c. injection of adriamycin or purimycin (at various doses) and the rats were followed for 7 days - 21 days. Development of severe nephrotic syndrome was established by monitoring the total body weight, urinary protein excretion (proteinuria) and at time of ending the experiment the volume of fluid in the abdominal cavity was determined (rats develop severe water retention and ascites). The effect of α-MSH or epoetin or α-MSH combined with epoetin was determined by treatment with the compounds for the initial 3 days after adrimycin or purimycin administration.

Experimental protocols

The following protocols were performed:

Protocol I-1: This protocol included 1) Rats with LPS induced lung inflammation for 24 hours (n=2) and 2) control rats (n=2).

Protocol I-II: This protocol included 1) Rats with LPS induced lung inflammation for 24 hours which were divided into the following treatment groups: 1) rats were treated with saline at onset of LPS induction and after 12 hours (n=6), 2) rats were treated with α-MSH (Phoenix Pharmaceutical Inc, Mountain View, CA, 50μg, i.v.) at onset of LPS induction and after 12 hours (n=6), 3) rats were treated with Epoitin (100 U/kg/day i.p.) at LPS induction and after 12 hours and 4) rats treated with a combination of epoitin (100 U/kg/day i.p.) and α-MSH (50μg, i.v.) at the onset of LPS and 12 hours later.

Protocol II: Systemic LPS-infusion:

II-1. Controls. Untreated rats without LPS infusion.

II-2 Vehicle: Rats receiving LPS infusion treated with 0.5 ml 0.9 % NaCl twice daily.

II-3 rh-EPO: Rats receiving LPS infusion treated with 200 I.U. epoitin alpha (EPO)/kg body weight in 0.5 ml 0.9% NaCl twice daily.

II-4 α-MSH: Rats receiving LPS infusion treated with 200 µg α-melanocyte stimulating hormone (α-MSH)/kg body weight in 0.5 ml 0.9% NaCl twice daily.

II-4 α-MSH+rh-EPO: Rats receiving LPS infusion treated with 200 µg α-MSH/kg body weightand 200 I.U.EPO/kg body weight in 0.5 ml 0.9% NaCl twice daily.

For protocols II-3, II-4 and II-5 the compounds (α-MSH and/or rh-EPO) were given i.v.. The first injections were given 12 hours after the implantation of the osmotic minipump.

Protocol III. Obstruction of the urinary tract

Protocol III-1: This protocol included 1) Rats with BUO for 24 hours (n=20) and 2) sham-operated rats (n=10). The BUO animals were divided into two groups: α-MSH nontreated (n=10) and α-MSH treated (n=10). α-MSH (Phoenix Pharmaceutical Inc, Mountain View, CA, 50µg, i.v.) was given at the onset of BUO and 12 hours later.

Protocol III-2: This protocol included 1) Rats with BUO for 24 hours and followed by release for 5 hours (n=10) and 2) Sham operated rats (n=5). The rats with BUO-R were divided into two groups: α-MSH nontreated (n=5) and α-MSH treated (n=5). α-MSH (50±g, i.v.) was given at the onset of BUO, 12 hours later and at the onset of release.

Protocol III-3: This protocol included 1) Rats with BUO for 24 hours and followed by release for 48 hours. The rats were divided into two groups: α-MSH nontreated (n=10) and α-MSH treated (n=13). α-MSH (50µg) was given with micro-osmotic pump via jugular vein at the onset of BUO. 2) Sham operated rats (n=8) treated with vehicle with micro-osmotic pump.

Protocol III-4: This protocol included rats, which had a detailed examination of renal function before onset and after release of BUO. 1) Rats with BUO for 24 hours followed by release for 48 hours (n=10) and 2) sham-operated rats (n=5). The BUO animals were divided into two groups: α-MSH nontreated (n=5) and α-MSH treated (n=5). α-MSH (Phoenix Pharmaceutical Inc, Mountain View, CA, 50µg, i.v.) was given at the onset of BUO and 12 hours later.

Protocol III-5: This protocol included 1) Rats with UUO for 24 hours (n=11) and 2) sham-operated rats (n=5). The UUO animals were divided into two groups: α-MSH nontreated

(n=5) and α-MSH treated (n=6). α-MSH (Phoenix Pharmaceutical Inc, Mountain View, CA, 50μg, i.v.) was given at the onset of UUO and 12 hours later.

Protocol III-6: This protocol included 1) Rats with BUO for 24 hours and 2) sham-operated rats. The BUO animals were divided into 4 groups: Non-treated rats (n=4), α-MSH treated (n=4)(α-MSH (Phoenix Pharmaceutical Inc, Mountain View, CA, 50μg, i.v.) was given at the onset of BUO and 12 hours later), Epoitin treated rats (n=4)(100 U/kg/day i.p.) and rats treated with a combination of epoitin (100 U/kg/day i.p.) and α-MSH (50μg, i.v.) was given at the onset of BUO and 12 hours later.

Protocol IV: Experimental nephritic syndrome.

Protocol IV-1. Rats were treated with adriamycin (7.5 mg/kg i.p.; n=12) and followed for 21 days, respectively. Shams received saline i.p. (n=6). Half of the adriamycin treated animals received α-MSH in osmotic minipumps (50 ug/day) during the entire experiments. The other half received vehicle (saline) in osmotic minipumps. In another series of experiments α-MSH treatment was administered (at the time of adriamycin injection and 6, 24 and 48 hours after adriamycin injection).

Protocol IV-2. Rats were treated with purimycin (100 mg/kg i.p., n=6) and followed for 11days, respectively. Half recieved α-MSH treatment (at the time of PAN injection and 6, 24 and 48 hours after PAN injection).

Protocol IV-3. Rats were treated with adriamycin (7.5 mg/kg i.p.; n=8) and followed for 21 days, respectively. Shams received saline i.p. (n=6). Half of the adriamycin treated animals received epoetin (100 units/kg/day) during the entire experiments. The other half received vehicle (saline).

Protocol IV-4. Rats were treated with purimycin aminoglycoside (100 mg/kg i.p., n=6) and followed for 11 days, respectively.Half recieved epoetin (100 units/kg/day).

Protocol IV-5. Rats were treated with purimycin (100 mg/kg i.p., n=6) and followed for 11days, respectively.Half recieved α-MSH treatment (at the time of PAN injection and 6, 24 and 48 hours after PAN injection) and in addition they received epoetin (100 units/kg/day).

Operative procedures:

Catheterisation of the Jugular Vein

The jugular vein was exposed at least 1 cm. The tip of the catheter was inserted into the vein and pushed forwards towards the heart (about 2.5cm) filled with a heparin-saline

solution ("heparin lock"). The catheter was then tied into the blood vessel with the ligature. With a small incision made in the dorsal nape of the neck the catheter was passed subcutaneously from the site of the entry of the catheter of the jugular vein to the dorsal incision down the side of the neck to emerge anterior. And a micro-osmotic pump was connected with the end of catheter.

Permanent bladder catherization

Catheters were permanently placed in the bladder for urine collection. One week before the experiment, the animals were anesthetized with halothane/N_2O. Using aseptic surgical techniques, sterile Tygon™ catheters (Norton Performance Plastics, Arkon, OH) were advanced into the abdominal aorta and the inferior vena cava via the femoral vessels. A sterile chronic suprapubic catheter was implanted into the bladder. After instrumentation, the rats were infused with saline subcutaneously (5 ml) and given a long-acting analgesic, Buprenorphinum (Temgesic™ ; Reckitt & Colman, Hull, United Kingdom), subcutaneously and housed individually. After a recovery period of 5 to 6 d, the rats were acclimatized to restriction by daily training sessions in restraining cages. The duration of each daily session was gradually increased from 1 to 3 h a day.

Primary Antibodies

For semiquantitative immunoblotting, previously characterized mouse monoclonal and affinity purified rabbit polyclonal antibodies were used:

1) AQP2 (LL127 1:6000): An affinity purified polyclonal antibody to AQP2

2) AQP1 (LL266 1:3000): An affinity purified polyclonal antibody to AQP1

3) AQP3 (LL178 1:400): An affinity purified polyclonal antibody to AQP3.

4) AQP4 (LL182AP): An affinity purified polyclonal antibody to AQP4 has previously been characterized.

4) NHE-3 (LL546AP): An affinity purified polyclonal antibody to NHE-3 has previously been characterized.

5) NaPi-2 (LL696AP): An affinity purified polyclonal antibody to type II Na-Pi cotransporter (NaPi-2) which was raised against the final 24 amino acids of COOH-terminal sequence has previously been characterized.

6) Na,K-ATPase: A monoclonal antibody against the α-1 subunit of Na,K-ATPase has previously been characterized.

7) BSC-1 (LL320AP): An affinity purified polyclonal antibody to the apical Na-K-2Cl cotransporter of the thick ascending limb has previously been characterized.

8) TSC (LL573AP): An affinity purified polyclonal antibody to the apical thiazide-sensitive Na-Cl cotransporter of the distal convoluted tubule has previously been characterized.

Clearance studies

Weight, water intake, food intake and urine output were observed during the rats were maintained in the metabolic cages. Urine was collected over 24-h periods throughout the study. Urine volume, osmolality, creatinine, sodium and potassium concentration were measured. Plasma was collected from abdominal aorta at the time of sacrifice for measurement of sodium and potassium concentration, creatinine, and osmolality. In protocols 4 and 9 detailed examinations of renal function was performed: The experiments were carried out between 8 a.m. and 1 p.m. The rats were transferred to a restraining cage and connected to infusion pumps via the vein catheter and to a BP transducer via the arterial catheter. Urine was collected in three periods of 20 min preceded by an equilibration period of 105 min. Throughout the experiment, a half isotone saline (77 mM NaCl) was infused at a rate of 70 µl/min to maintain a minimum urine flow necessary for accuracy of the bladder emptying. ^{14}C-tetraethylammonium bromide (0.83 µCi/ml ; New England Nuclear, Boston, MA), together with ^{3}H-inulin (2.5 µCi/ml ; Amersham, Rainham, United Kindgom) and LiCl (13 mmol/L), were infused together with the saline as markers of effective renal plasma flow (ERPF), GFR, and tubular fluid delivery from proximal tubules (V_{prox}), respectively. A bolus of markers four times the continuous infusion velocity was given in the first 15 min. Blood samples (200 µl) were drawn from the arterial catheter after 105 and 165 min. Blood substitution with donor blood was given after each blood sample. Mean arterial BP was recorded continuously using a Uniflow™ transducer (Baxter, Irvine, CA) connected to a preamplifier and PC registration. Clearance experiments were carried out in BUO and SHAM rats 7 days prior to obstruction and 48 hours after release of BUO.

Analysis

Urine volume was determined by gravimetric means. Li^+ concentration was determined in plasma and urine by flame emission photometry and atomic absorption spectrophotometry, respectively. ^{14}C-tetraethylammonium (TEA) and ^{3}H-inulin in plasma and urine were determined by dual label liquid scintillation counting (Wallac™ model 1409; Helsinki, Finland). Sample (15 µl) and 285 µl of water were mixed with 2.5 ml of scintillation liquid (Ultima Gold™ ; Packard Instruments, Meriden, CT). Correction of dpm was performed by automatic efficiency control.

Calculations

Renal clearances (*C*) were calculated by the standard formula:

$C = U \times V/P$

where *U* is urine concentration, *V* is urine flow rate, and *P* is plasma concentration.

In previous studies, the renal extraction fraction of TEA has been shown to approximate 90%, and the validity of TEA as an estimate of ERPF has previously been documented. By use of C_{TEA}, C_{IN}, and C_{LI}, the following parameters were calculated:

ERPF = Effective renal plasma flow (CTEA)

GFR = Glomerular filtration rate (Cin)

Membrane fractionation for immunoblotting

Inner medulla and whole kidneys were homogenized (0.3 M sucrose, 25 mM imidazole, 1 mM EDTA, pH 7.2, containing 8.5 µM leupeptin. 1 mM phenylmethyl sulfonylfluoride) using an ultra-turrax T8 homogenizer (IKA Labortechnik, Germany), at 5 strokes for 20 seconds (inner medulla) or at 5.5 strokes for 30 seconds (whole kidney) and the homogenate was centrifuged in an Eppendorf centrifuge at 4000 g for 15 minutes at 4°C to remove whole cells, nuclei and mitochondria. Gel samples (Laemmli sample buffer containing 2% SDS) were made of this pellet.

Electrophoresis and immunoblotting

Samples of membrane fractions from inner medulla and whole kidney were run on 12% or 8-16% gradient polyacrylamide minigels (Bio-Rad Mini Protean II) for AQP1, AQP2 and AQP3 (or other renal transporters). For each gel, identical gel was run in parallel and subjected to Coomassie staining to assure identical loading. Then gels were subjected to immunoblotting. After transfer by electroelution to nitrocellulose membranes, blots were blocked with 5% milk in PBS-T (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, 0.1% Tween 20, pH 7.5) for 1 h and incubated overnight at 4°C with affinity-purified primary antibodies (see above). The labeling was visualized with horseradish peroxidase (HRP)-conjugated secondary antibodies (P448, DAKO, Glostrup Denmark, diluted as 1:3000) using an enhanced chemiluminescence system (ECL, Amersham International, UK).

Quantitation of total kidney levels of AQPs and other renal transporters.

ECL films with bands within the linear range were scanned using an AGFA scanner (ARCUS II) and Corel Photopaint Software to control the scanner. For AQP1 and AQP2,

both the 29-kDa and the 35- to 50-kDa bands (corresponding to nonglycosylated and the glycosylated species) were scanned. For AQP3, both the 27-kDa and the 33- to 40-kDa bands (corresponding to nonglycosylated and the glycosylated species) were scanned. The labeling density was determined of blots where samples of kidneys from α-MSH treated and nontreated groups were run together with samples from control kidneys. The labeling density was corrected by densitometry of coomassie stained gels. (i.e. to control for minor difference in loading)

Quantitation of urinary protein excretion

To determine the levels of proteinuria in experimental nephrotic syndrome urine was examined daily using Albym (Boehringer-Mannheim).

Statistical analysesValues were presented in the text as means ± standard errors. Comparisons between groups were made by unpaired t-test. *P* values < 0.05 were considered significant.

Results

LPS inhalation for 20 min is associated with a marked inflammatory response in the lung tissue

LPS induced a dramatic influx of inflammatory cells in the lung tissue. Differential counting revealed that lung tissue exposed for LPS had large numbers of neutrophil leucocytes (68.5±8%), eosinofiles (8.6±4 %), lymphocytes (15.3 ±6%), and monocytes (8±2%) (Table 1).

α-MSH prevents influx of inflammatory cells in lung tissue exposed with LPS

Treatment with α-MSH reduced the influx of inflammatory cells in the lung tissue exposed with LPS. Differential counting revealed that the number of eosinofils were reduced (1.8±1.3% vs. 3.6±2.7%), and neutrophil granulocytes were markedly reduced (10.6±10.6% vs. 34.4±26.1%) (Table 1).

Epoitin prevents influx of inflammatory cells in lung tissue exposed with LPS

Treatment with epoitin reduced the influx of inflammatory cells in the lung tissue exposed with LPS. Differential counting revealed that the number of eosinofils were reduced

(0.7±0.6% vs. 3.6±2.7%), and neutrophil granulocytes were markedly reduced (10.9±12.7% vs. 34.4±26.1%) (Table 1).

Combined treatment with α-MSH and epoitin almost completely prevented the influx of inflammatory cells in lung tissue exposed with LPS

Combined treatment with α-MSH and epoitin dramatically reduced the influx of inflammatory cells in the lung tissue exposed with LPS. Differential counting revealed that the number of eosinofiles were reduced (0.6±0.51% vs. 3.6±2.7%), and neutrophile granulocytes were markedly reduced (3.8±1.9% vs. 34.4±26.1%) (Table 1). In addition the influx of monocytes was also significantly prevented with this treatment (6.0±4.1% vs. 13.9±8.4%) (Table 1).

LPS administration in osmotic minipumps for 3 days is associated with a severe inflammatory response and severe renal failure

The rats were followed for three days after the implantation of the osmotic minipump with LPS. Glomerular filtration rate as evaluated through creatinine clearance was significantly reduced with 68% in the LPS-Vehicle group compared to the untreated control rats. Thus the animals had severe renal failure. The mortality was 20%.

Treatment with α-MSH or with epoitin or with combined α-MSH and epoitin prevented the inflammatory-induced renal failure seen in response to LPS administration

During 3 days of LPS-administration the mortality in the vehicle treated group were 20% (1 out of 5). All animals survived in the other groups, i.e. rh-EPO treatment, α-MSH treatment; combined treatment with low dose rh-EPO and α-MSH, and in the sham-operated controls. The parameters of renal function and other parameters are described in Table 2. Glomerular filtration rate as evaluated through creatinin clearance was significantly reduced with 68% in the LPS-Vehicle group compared to the untreated control rats. Both rh-EPO and α-MSH treatment increased GFR in the LPS treated rats by 51% and 57%, respectively. However GFR was still severely affected compared to the untreated control rats. The combined treatment with rh-EPO and α-MSH significantly increased GFR by 111% compared to the LPS-Vehicle group suggesting a synergestic effect of the combined treatment with rh-EPO and α-MSH.

BUO for 24 hours, 5 and 48 hours after release of BUO are associated with reduced AQP1, AQP2 and AQP3

As previously studied, immunoblotting revealed that 24 h of BUO and 48 hours after release of BUO were associated with a significant downregulation of AQP-2 expression compared with sham-operated controls (13±4%). Semiquantitative immunoblotting from the inner medulla of rats with 24h BUO and BUO-R for, 5 and 48 hours revealed that AQP3 expression was persistently downregulated. Both AQP3 bands (the 27-kDa and 33- to 40-kDa bands) were decreased proportionately. Densitometric analysis revealed a significant decrease in AQP3 expression in rats with 24 h BUO to 19±4% of sham levels (100±7%, $p<0.05$). Furthermore, AQP3 protein levels were marked decreased at 5 hours after release to 15±5% of sham levels (100±9%, $p<0.05$) and at 48 hours after release to 10±5% of sham level (100±1%, $p<0.05$).

Semiquantitative immunoblotting using membrane fractions prepared from the whole kidney of rats with 24h BUO and BUO-R for 5 and 48 hours revealed that AQP1 expression was persistently downregulated. Both AQP1 bands (the 29-kDa and 35- to 50-kDa bands) were decreased proportionately. Densitometric analysis revealed a significant decrease in AQP1 expression in rats with 24 h BUO to 53±7% of sham levels (100±9%, $p<0.05$). Furthermore, AQP1 protein levels remained markedly decreased at 5 hours after release of BUO to 7 ±2% of sham levels (100±22%, $p<0.05$) and at 48 hours after release to 30±5% of sham level (100±10%, $p<0.05$).

BUO for 24 hours, 5 and 48 hours after release of BUO are associated with downregulation of Na,K-ATPase

Semiquantitative immunoblotting using membrane fraction prepared from whole kidney of rats with 24 h BUO, and BUO followed by 5 h and 48 h after release of BUO showed a persistant downregulation of Na,K-ATPase to 35 - 50% of control levels.

α-MSH partially prevents AQP2 and AQP3 downregulation in response to 24 h of BUO

Semiquantitative immunoblotting using membrane fractions prepared from the inner medulla of rats with 24 h BUO and sham operated control rats revealed that α-MSH treatment significantly increased AQP2 expression were compared with nontreated rats (38±5% vs. 13±4%, $p<0.05$). At the same time, immunoblotting also showed that

expression of AQP3 in rats with 24 h BUO was significantly upregulated in response to α-MSH treatment compared with nontreated rats (44±3% vs. 19±4%, $p<0.05$).

Plasma concentrations data showed that in α-MSH treated rats with 24 h BUO plasma sodium levels had a marked increase compared with 24 h BUO rats without α-MSH treatment from 135±2mmol/L to 139±0.6 mmol/L. There was no difference between α-MSH treated rats and sham operated rats.

α-MSH prevents AQP3 and AQP1 downregulation in response to 24 h BUO followed by release for 5 hours

Semiquantitative immunoblotting using membrane fractions prepared from the inner medulla of 24 h BUO rats followed by release for 5 hours and sham operated rats revealed that with α-MSH treatment AQP3 expression was significantly increased compared with nontreated rats with 24 h BUO followed by release for 5 hours form 14±5% to 34±4% of sham level(100±14%).

Semiquantitative immunoblotting using membrane fractions prepared from whole kidney of rats with 24 h BUO followed by release for 5 hours and sham operated control rats revealed that with α-MSH treatment significantly increased AQP1 expression levels compared with nontreated BUO-R rats (81±21% vs. 7±2%, $p<0.05$) (Fig 2 A and B). Furthermore, immunoblotting prepared from outer medulla and cortex also showed the same result that in α-MSH treated rats AQP1 expression was a marked increase significantly compared with nontreated rats from 35±2% to 62±9% of sham level.

α-MSH prevents AQP1 downregulation in response to 24 h BUO followed by release for 48 hours

In rats with 24 h BUO and 5 hours after release of BUO, α-MSH was given every 12 hours. In rats with 48 hours after release of BUO, α-MSH was given with micro-osmotic pump continuously. Semiquantitative immunoblotting using membrane fractions prepared from the whole kidney of 24 h BUO rats followed by release for 48 hours and sham operated rats revealed that with α-MSH treatment the levels of AQP1 expression were significantly increased compared with nontreated rats with 48 hours after release of BUO form 24±5% to 58±6% of sham level (100±10%). Furthermore, immunoblotting prepared from outer medulla and cortex also showed the same result that in α-MSH treated rats

AQP1 expression was a marked increase compared with nontreated rats to 73±8% of sham level.

α-MSH completely normalise expression of Na,K-ATPase during BUO and 5 and 48 hours after release of BUO

Semiquantitative immunoblotting using membrane fractions prepared from whole kidney of rats with 24 h BUO showed that Na,K-ATPase levels were completely normalized. Also, 24 h BUO followed by 5 h of release and sham operated control rats revealed that α-MSH completely normalized Na-K-ATPase levels (102±14%). α-MSH treatment also completely normalized Na-K-ATPase levels 48 hours after release of BUO (114±10%).

Treatment with α-MSH prevents the increase in plasma creatinine and restores GFR and RPF in response to 24 hours of BUO followed by 48 hours of release

Plasma creatinine is an important marker of glomerular filtration rate. In rats with BUO for 24 h followed by 48 of release and sham operated control rats α-MSH treatment significantly prevented the dramatic increase in plasma creatinine (indicating severe renal insufficiency). Plasma creatinine levels were significantly reduced in α-MSH treated rats (79±34 μmol/l vs.160±34 μmol/l, $p<0.05$,). In addition, plasma urea which is another important marker of renal function almost normalized in rats treated with α-MSH (13±2 mmol/l vs. 35±9 mmol/l, $p<0.05$). Also GFR and ERPF were completely normalized (Fig 3 A and B).

Epoitin partially prevents AQP2 downregulation in response to 24 h of BUO

Semiquantitative immunoblotting using membrane fractions prepared from the inner medulla of rats with 24 h BUO and sham operated control rats revealed that epoitin treatment significantly increased AQP2 expression were compared with nontreated rats (30±18% vs. 16±3%, $p<0.05$).

Preventive effect of α-MSH, or Epoetin treatment on adriamycin and purimycin-induced nephrotic syndrome in rats.

Treatment with α-MSH for 3-4 days after i.p injection of adriamycin (Protocol IV-1) significantly prevented the ascites production by 89 ± 7% indicating a marked preventive effect of α-MSH on experimentally induced nephrotic syndrome. A similar dramatic effect was also observed in low dose purimycin treatment (80±8% reduction, n=6) and a marked

reduction in proteinuria (Protocol IV-2). A less potent effect was seen in very servere purimycin (high dose) induced nephrotic syndrome. EPO treatment once every 24 hours also prevented the ascites formation in adriamycin-induced nephrotic syndrome and co-treatment of epoetin and α-MSH produced an even greater effect.

5

TABLES

Table 1.

A) Differential counting showed that LPS induced a marked inflammatory response in the

B) Differential counting showed marked reduction in the number of inflammatory cells with all three treatment regimens. Importantly the data show a dramatic reduction in response to combined treatment with α-MSH+Epoitin.

A	Differential counting of cells %			
Treatment	Eosinofiles	Neutrofiles	Lymfocytes	Monocytes
Controls	0	0	0	0
LPS	8.6±4	68.5±8	15.2 ±6	8±2

B	Differential counting of cells %			
Treatment	Eosinofiles	Neutrofiles	Lymfocytes	Monocytes
Vehicle	3.6±2.7	34.4±26.1	48.5±20.9	13.9±8.4
α-MSH	1.8±1.3	10.6±10.6	72.3±12.1	12.3±7.4
Epoitin	0.7±0.6	10.9±12.7	77.1±9.9	11.4±4.6
α-MSH+Epoitin	0.6±0.51	3.8±1.9	89.4±4.1	6.0±4.1

Table 2. Effects of vehicle (0.9% NaCl); rh-EPO (200 U/kg b.w.); α-MSH (200 µg/kg b.w.) and the combination of rh-EPO (200 U/kg b.w.) and α-MSH (200 µg/kg b.w.) on renal function in rats treated with continious LPS infusion (200 µg/kg/h) during three days.

	Creatinin Clearance ml/min
Control:	1.11 ± 0.08
Vehicle:	0.35 ± 0.07
rh-EPO:	0.53 ± 0.06 *
α-MSH:	0.55 ± 0.05 *
α-MSH+rh-EPO:	0.74 ± 0.06 * # ¤:

*: different from Vehicle treated rats; $p<0.05$; #: different from rh-EPO treated rats; $p<0.05$; ¤: different from α-MSH treated rats; $p<0.05$.

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CLAIMS

1. Method for treatment or prevention of a non-ischemic condition in one or more organ(s) or tissue(s), the method comprising administering of an effective dosage of α-MSH and/or of an α-MSH equivalent and/or a dosage of EPO and/or an EPO equivalent to the individual in need thereof.

2. Method according to claim 1 wherein the dosage of α-MSH and/or of an α-MSH equivalent and/or EPO and/or an EPO equivalent is administered prophylactically for preventing the establishment or progress of the condition, or of any symptom of the condition.

3. Method for treatment or prevention of an inflammatory condition in one or more organ(s) or tissue(s), the method comprising administering of an effective dosage of α-MSH and/or of an α-MSH equivalent and/or a dosage of EPO and/or an EPO equivalent to the individual in need thereof.

4. Method according to claim 3 wherein the dosage of α-MSH and/or of an α-MSH equivalent and/or EPO and/or an EPO equivalent is administered prophylactically for preventing the establishment or progress of the condition, or of any symptom of the condition.

5. Method according to claim 1 or 3 wherein the dosage of α-MSH and/or of an α-MSH equivalent and EPO and/or an EPO equivalent is administered as a single dosage, regular or continued administration, or as a sequential administration.

6. Method according to claim 1 or 3 wherein condition is caused by an infection.

7. Method according to claim 1 or 3 wherein the condition is caused by a cancer or a by premalignant disorder.

8. Method according to claim 1 or 3 wherein the α-MSH equivalent is a substance acting on the α-MSH receptor and/or on the melanocortin receptor.

9. Method according to claim 1 or 3 wherein the treatment or prevention comprises administration of a dosage unit of EPO and/or an EPO equivalent.

10. Method according to claim 1 or 3 wherein a combination of α-MSH and/or α-MSH equivalent with EPO and/or an EPO equivalent is adminstered.

11. Use of α-MSH and/or an equivalent of α-MSH and/or EPO and/or an EPO equivalent for the preparation of a medicament for treatment or prevention of a non-ischemic condition.

12. A pharmaceutical composition comprising a unit dosage of EPO and/or EPO equivalent and a unit dosage of α-MSH and/or of an α-MSH equivalent together with a suitable pharmaceutical carrier.

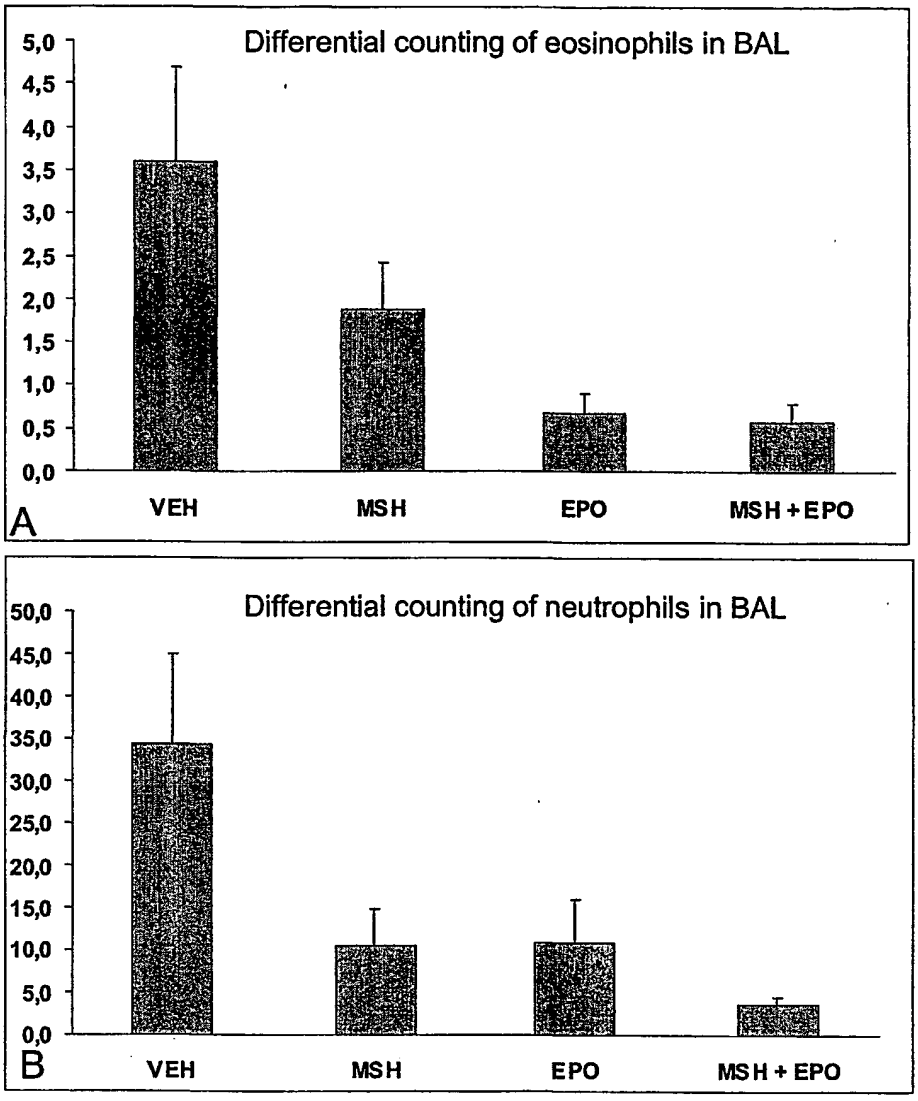
Differential counting of eosinophils in BAL
5,0
4,5
4,0
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
A
VEH
MSH
EPO
MSH + EPO
Differential counting of neutrophils in BAL
50,0
45,0
40,0
35,0
30,0
25,0
20,0
15,0
10,0
5,0
0,0
B
VEH
MSH
EPO
MSH + EPO

Fig. 1

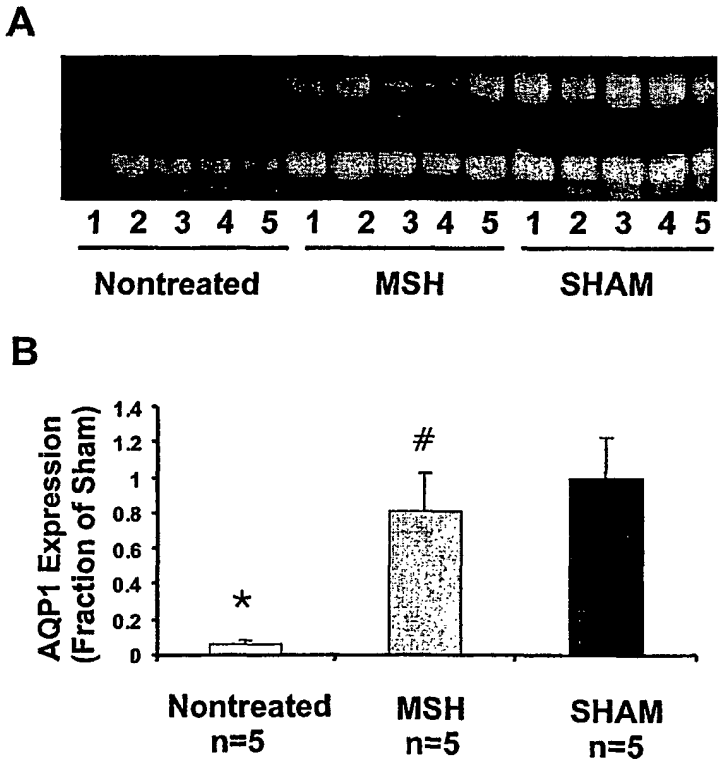
A
1 2 3 4 5 1 2 3 4 5 1 2 3 4 5
Nontreated
MSH
SHAM
B
AQP1 Expression (Fraction of Sham)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
#
Nontreated n=5
MSH n=5
SHAM n=5

Fig. 2

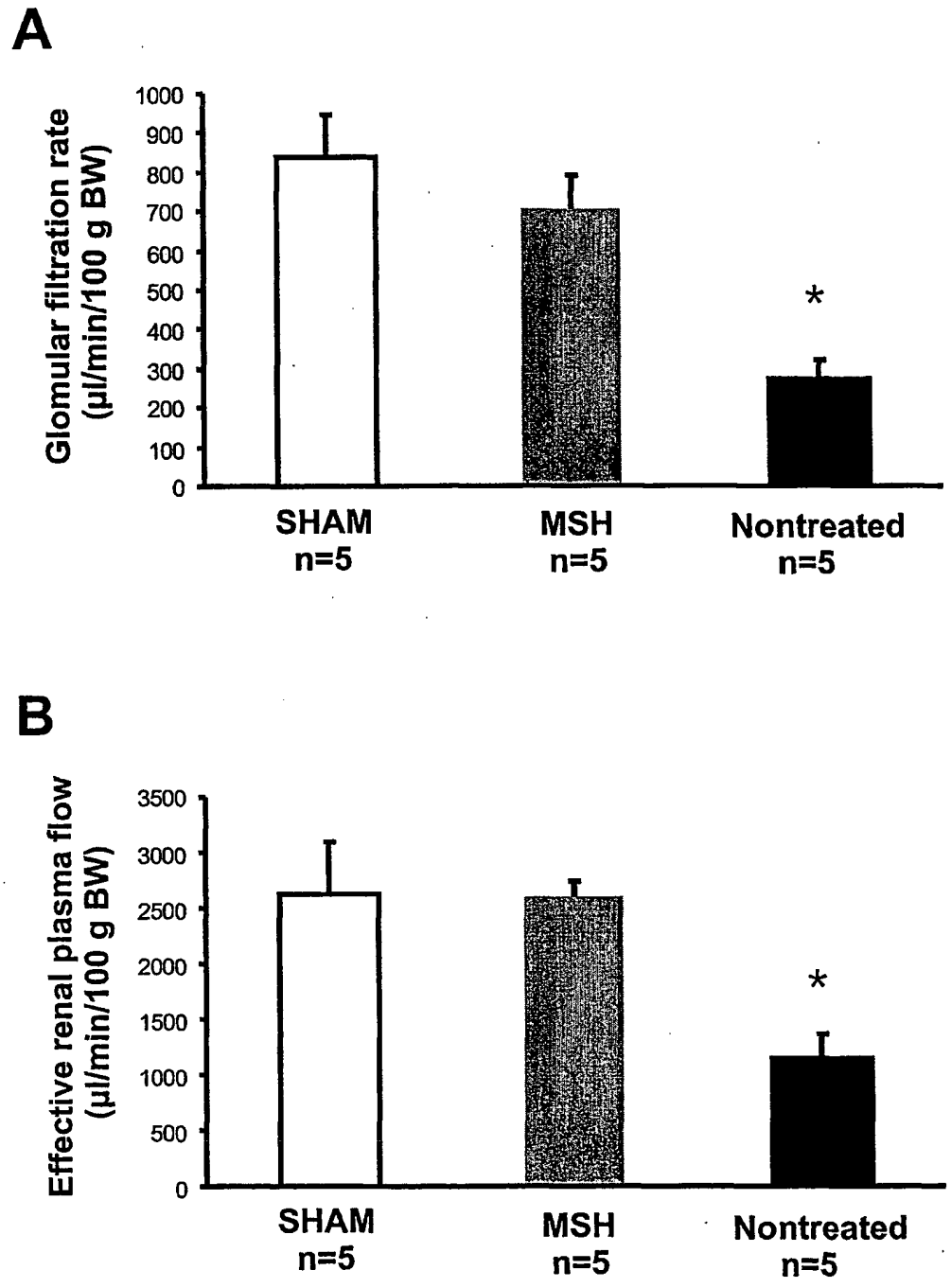
A
Glomular filtration rate (µl/min/100 g BW)
1000
900
800
700
600
500
400
300
200
100
0
*
SHAM n=5
MSH n=5
Nontreated n=5
B
Effective renal plasma flow (µl/min/100 g BW)
3500
3000
2500
2000
1500
1000
500
0
*
SHAM n=5
MSH n=5
Nontreated n=5

Fig. 3